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A1

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :

C12N 15/11, A61K 31/70

A1

(11) International Publication Number:

WO 98/54315

(43) International Publication Date: 3 December 1998 (03.12.98)

(21) International Application Number: PCT/US98/10001

(22) International Filing Date: 15 May 1998 (15.05.98)

(30) Priority Data:
08/867,230 30 May 1997 (30.05.97) US(71) Applicant: RESEARCH CORPORATION TECHNOLOGIES,
INC. [US/US]; Suite 600, 101 North Wilmot Road, Tucson,
AZ 85711-3335 (US).(72) Inventor: PETRYSHYN, Raymond, A.; 5756 Keyser Road,
Hume, VA 22639 (US).(74) Agents: DIGIGLIO, Frank, S. et al.; Scully, Scott, Murphy &
Presser, 400 Garden City Plaza, Garden City, NY 11530
(US).(81) Designated States: CA, JP, European patent (AT, BE, CH, CY,
DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT,
SE).**Published***With international search report.
Before the expiration of the time limit for amending the
claims and to be republished in the event of the receipt of
amendments.*

(54) Title: CELL GROWTH-CONTROLLING OLIGONUCLEOTIDES

(57) Abstract

The present invention provides a partial cDNA corresponding to an RNA containing double stranded regions (R-RNA), which, when transcribed *in vitro*, gives rise to an RNA transcript that activates PKR. An approximately 226-252 bp nucleotide (nt) sequence responsible for activation of PKR (the activation sequence) has been identified within the cDNA and isolated. Antisense oligonucleotides corresponding to specific portions of the 252 nt cDNA fragment stimulate proliferation of different cells in culture. Various portions of the cDNA or R-RNA may also be used to inhibit cell proliferation in cell cultures. The present invention further provides pharmaceutical compositions comprising the subject nucleic acid fragments and oligonucleotides. Kits which comprise at least one of the subject isolated nucleic acid molecules or oligonucleotides and a pharmaceutically acceptable carrier are also provided.

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CELL GROWTH-CONTROLLING OLIGONUCLEOTIDES

1 PKR is a dsRNA-dependent (double stranded
RNA-dependent) protein kinase which is implicated in
the regulation of several cellular processes including
cell proliferation. Previous studies using embryonic
5 mouse 3T3-F442A cells have indicated that PKR
undergoes phosphorylation and activation *in vivo*.
This activation of PKR has been attributed to a subset
of poly(A)⁺- rich cellular RNA having sufficient
secondary structure to interact with the kinase. The
10 present invention provides a partial cDNA
corresponding to the RNA containing double stranded
regions, which, when transcribed *in vitro*, gives rise
to an RNA transcript that activates PKR. An
approximately 226-252 bp nucleotide (nt) sequence
15 responsible for activation of PKR (the activation
sequence) has been identified within the cDNA and
isolated. Antisense oligonucleotides corresponding to
specific portions of the 226 nt cDNA fragment
stimulate proliferation of human hematopoietic cells
and mouse fibroblasts in culture.

20 The dsRNA-dependent eIF-2 α kinase (hereinafter
"PKR"), also known as DAI, p68, dsI and dsRNA-PK
(Clemens, et al., 1993, *J. Interferon Res.* 13: 241),
is an interferon (IFN)-induced enzyme that mediates,
25 in part, the antiviral and antiproliferative effects
of IFN (Pestka, et al., 1987, *Ann. Rev. Biochem.* 56:
727-777; Hovanessian, et al., 1989, *J. Interferon Res.*
9: 641-647). Other studies have indicated that PKR
may also be involved in the regulation of cell growth
30 and differentiation of some cells, function as a tumor

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1 suppressor (Meurs, et al., 1993, *Proc. Natl. Acad. Sci.*
90: 232-236; Petryshyn, et al., 1994, *J. Biol. Chem.*
259: 14736-14742; Petryshyn, et al., 1988, *Proc. Natl.*
Acad. Sci. 85: 1427-1431; Judware, et al., 1991, *Mol.*
Cell. Biol. 11: 3259-3267; Koromilas, et al., 1992,
5 Science 257: 1685-1689; Chong, et al., 1992, *EMBO J.*
11: 1553-1562) and modulate signal transduction
(Kumar, et al., 1994, *Proc. Natl. Acad. Sci.* 91: 6288-
6292; Maran, et al., 1994, *Science* 265: 789-792).

Still other studies have indicated that PKR
may be involved in regulating programmed cell death
10 (apoptosis) (Young et al., 1996 *Proc. Natl. Acad. Sci.*
93:12451-12455). The mechanism by which PKR controls
protein synthesis *in vitro* is reasonably understood
(Lebleu, et al., 1976, *Proc. Natl. Acad. Sci.* 73:
3107-3111; Farrell, et al., 1977, *Cell* 11: 187-200;
15 Levin, et al., 1978, *Proc. Natl. Acad. Sci.* 75: 1121-
1125; Samuel, 1979, *Proc. Natl. Acad. Sci.* 76: 600-
604; Petryshyn, et al., 1983, *Methods Enzymol.* 99:
346-362). In the presence of ng/ml levels of dsRNA,
ATP and divalent cations, the enzyme undergoes
20 autophosphorylation which converts it from a latent to
an active protein kinase (Hovanessian, 1989, *J.*
Interferon Res. 9: 641-647; Lebleu, et al., 1976).
The autophosphorylation and activation of PKR is
prevented by high concentrations of dsRNA (Farrel, et
25 al., 1977; Hunter, et al., 1975, *J. Biol. Chem.* 250:
409-417). Upon activation, the kinase catalyzes the
phosphorylation of the α -subunit (38 kDa) of the
eukaryotic initiation factor 2 (eIF-2 α) (Lebleu, et
al., 1976; Farrel, et al., 1977; Levin, et al. 1978;
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Samuel, 1979; Petryshyn, et al., 1983). The phosphorylation of eIF-2 α prevents the ability of eIF-2 to exchange GDP for GTP which is catalyzed by eIF-2 β (Matts, et al., 1984, *J. Biol. Chem.* 259: 6708-6711; Rowlands, et al., 1988, *J. Biol. Chem.* 263: 5526-5533). This cascade of reactions results in the inhibition of protein synthesis (London, et al., 1987, *The Enzymes Vol. 18*; Hershey, 1989, *J. Biol. Chem.* 264: 20823-20826).

Duplex RNA molecules such as reovirus dsRNA and poly(I) \cdot poly(C) (PIC) are well established activators of PKR (Hovanessian, 1989), but the details of the activation process are not fully understood (Galabru, et al., 1989, *Eur. J. Biochem.* 178: 581-589; Kostura, et al., 1989, *Mol. Cell. Biol.* 9: 1576-1586; Kitajewski, et al., 1986, *Cell* 45: 195-200). Moreover, several single stranded (ss) viral RNAs including adenovirus VA1 RNA (Schneider, et al., 1987, *Ann. Rev. Biochem.* 56: 317-332; Furtado, et al., 1989, *J. Virol.* 63: 3423-3434; Ghadge, et al., 1994, *J. Virol.* 68: 4137-4151), Epstein Barr Virus EBER-1 RNA (Clarke, et al., 1991, *Nucleic Acids Res.* 19: 243-248) and HIV-1 mRNA (Edery, et al., 1989, *Cell* 56: 303-312; Sen Gupta, et al., 1989, *Nucl. Acids Res.* 17: 969-978; Roy, et al., 1991, *J. Virol.* 65: 632-640; Judware, et al., 1993, *J. Interferon Res.* 13: 153-160) have been demonstrated to contain secondary structures which interact and modulate the activity of PKR. Relatively little is known about the mechanism by which structural elements within these RNAs interact with the kinase but studies have indicated that the amino

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terminal portion of the protein is important for RNA binding (Feng, et al., 1992, *Proc. Natl. Acad. Sci.* 89: 5447-5451; Manche, et al., 1992, *Mol. Cell Biol.* 12: 5238-5248; Petryshyn, et al., 1994, *Progress in Molecular and Subcellular Biology Vol. 14*; Chong, et al., 1992). Of significance are the findings that eIF-2 α undergoes phosphorylation in response to addition of cytoplasmic mRNA (Baum, et al., 1983, *Biochem. Biophys. Res. Commun* 114: 41-49) or polysomal RNA (Pratt, et al., 1988, *Nucl. Acids Res.* 16: 3497-3510) prepared from uninfected cells and that this phosphorylation is prevented by high concentrations of poly(I) \cdot poly(C) (Baum, et al., 1983; Pratt, et al., 1988). In addition, altered levels of eIF-2 phosphorylation and PKR activity have been reported in some cells subjected to heat-stress conditions (Dubois, et al., 1991, *J. Biol. Chem.* 266: 9707-9711). Other studies have indicated that the mRNA for PKR itself is capable of facilitating the phosphorylation of PKR (Thomis, et al., 1993, *J. Virol.* 67: 7695-7700). These observations suggest that some cellular RNAs may regulate the activity of PKR and raise the possibility that the enzyme has a regulatory role in uninfected cells (Petryshyn, et al., 1994). This is supported by the finding of an accumulation of dsRNA capable of activating PKR in embryonal carcinoma cells that have been induced to differentiate but not in uninduced cells (Belkumeur, et al., 1993, *Mol. Cell Biol.* 13: 2846-2857). To date, however, the extent and nature of the cellular RNA(s) that mediate the

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activation of PKR in uninfected cells remain to be identified.

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The role of IFN and PKR in the regulation of growth and differentiation of mouse 3T3-F442A cells has recently been investigated. 3T3-F442A cells spontaneously produce and secrete IFN and exhibit a pattern of PKR phosphorylation which is related to specific stages of growth (Petryshyn, et al., 1984). PKR is phosphorylated both *in vivo* and *in vitro* in the absence of viral infection or added dsRNA (Petryshyn, et al., 1988). The phosphorylation of PKR is concomitant with increased phosphorylation of eIF-2 α , diminished eukaryotic initiation factor 2- β (eIF-2 β) activity and a marked reduction in protein synthesis (Petryshyn, et al., 1996, *Arch. Biochem. Biophys.* 328: 290-297).

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A subset of poly(A)⁺- rich cytoplasmic RNA that is responsible for activation of PKR in 3T3-F442A cells ("Regulatory RNA" or "R-RNA") has recently been isolated (Li, et al., 1991, *Eur. J. Biochem.* 195: 41-48) although the nature and exact number of RNAs comprising the R-RNA activity was not determined.

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The present invention provides a partial cDNA of about 850 base pairs corresponding to a single and specific cellular R-RNA which, when transcribed *in vitro*, gives rise to an RNA transcript which retains its property to activate PKR. In addition, the present invention provides a 226-252 nucleotide fragment of the partial cDNA which corresponds to that portion of R-RNA necessary for PKR activation ("the activation sequence"). An antisense molecule to the

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226-252 nt cDNA fragment as well as a number of
smaller antisense oligonucleotides all of which bind
to the sense strand of the R-RNA and thus prevent
activation of PKR are also provided. The present
invention further provides methods of stimulating the
proliferation of hematopoietic cells and fibroblast
cells in culture.

The present invention provides a portion of
a cDNA corresponding to a specific dsRNA known as
Regulatory RNA (R-RNA) which interacts with PKR
through its double stranded RNA regions and stimulates
autophosphorylation of PKR. The subject partial cDNA
molecule is approximately 847 nucleotides in length
and has the sequence set forth in SEQ ID NO:1. The
present invention also provides a 225-252 nt fragment
of the partial R-RNA cDNA, which is responsible for
the activation of PKR (herein referred to as the
activation sequence).

In one aspect of the invention, there is
provided an R-RNA from which the subject cDNA may be
reverse transcribed. The sequence of the subject R-
RNA is set forth in SEQ ID NO:2 and is useful in cell
cultures for purposes of inhibition of cell
proliferation (expansion). For example, the subject
R-RNA may be administered *ex vivo* to bone marrow cells
isolated from patients with hematological cancers.
After sufficient time, the bone marrow cells are
transplanted back into the donor patient.

In another aspect of the invention, there is
provided an antisense molecule corresponding to the
sense strand of the subject R-RNA. The subject

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antisense molecule binds to R-RNA, thereby interfering
with the activation of PKR by R-RNA. The subject
1 antisense molecule is therefore useful for preventing
the autophosphorylation of PKR and the inhibition of
cell division resulting from such autophosphorylation.

5 In one embodiment of the invention, there is
provided an isolated nucleic acid molecule consisting
of a nucleotide sequence or complementary to a
nucleotide sequence as set forth in SEQ ID NO:1 or
having at least 50% similarity or complementarity
thereto.

10 In another embodiment of the invention,
there is provided an isolated nucleic acid molecule
consisting of a nucleotide sequence or complementary
to a nucleotide sequence as set forth in SEQ ID NO:2
or having at least 50% similarity or complementarity
15 thereto.

The present invention further provides an
isolated nucleic acid molecule consisting of or
complementary to nucleotides 178-430 of SEQ ID NO:1 or
having at least 50% similarity or complementarity
20 thereto.

In another embodiment of the invention,
there is provided an isolated nucleic acid molecule
which hybridizes to the activation sequence of the R-
RNA i.e., nucleotides 178-430 of SEQ ID NO:2 under
25 medium to high stringency conditions.

In another embodiment, an isolated nucleic
acid molecule consisting of or complementary to
nucleotides 263-283 of SEQ ID NO:1 or having at least
50% similarity or complementarity thereto is provided.
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1 An isolated nucleic acid molecule which
hybridizes to nucleotides 263-283 of SEQ ID NO:2 under
medium to high stringency conditions is yet another
embodiment of the present invention.

5 In a further embodiment of the present
invention, there is provided an isolated nucleic acid
molecule consisting of or complementary to nucleotides
374-393 of SEQ ID NO:1 or having at least 50%
similarity or complementarity thereto.

10 In yet another embodiment of the invention,
there is provided an isolated nucleic acid molecule
which hybridizes to nucleotides 374-393 of SEQ ID NO:2
under medium to high stringency conditions.

15 In accordance with the present invention,
oligonucleotides complementary to specific portions of
the subject R-RNA sense strand are also provided. By
"specific portions" is meant specific nucleotide
sequences found within the R-RNA activation sequence.
The subject antisense oligonucleotides of the present
invention comprise at least eight or nine nucleotides
which are complementary to at least eight or nine
20 contiguous nucleotides of nucleotides 178-430 as set
forth in SEQ ID NOs:1 or 2. Other antisense
oligonucleotides include oligonucleotides comprising
at least eight or nine nucleotides which are
complementary to: at least eight or nine contiguous
25 nucleotides of nucleotides 263-283 as set forth in SEQ
ID NOs:1 or 2, or at least eight or nine contiguous
nucleotides of nucleotides 374-393 as set forth in SEQ
ID NOs: 1 or 2. Larger antisense oligonucleotides of,

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for example, 10 to greater than 25 nucleotides are also contemplated by the present invention.

1 In one embodiment of the invention, the antisense oligonucleotide comprises the nucleotide sequence set forth in SEQ ID NO:6. In another
5 embodiment of the invention, the antisense oligonucleotide comprises at least eight contiguous nucleotides derived from the sequence set forth in SEQ ID NO:6.

10 In another embodiment of the invention, the antisense oligonucleotide has the nucleotide sequence set forth in SEQ ID NO:7. In another embodiment of the invention, the antisense oligonucleotide comprises at least eight contiguous nucleotides derived from the sequence set forth in SEQ ID NO:7.

15 In yet another embodiment of the invention, the antisense oligonucleotide has the nucleotide sequence set forth in SEQ ID NO:10. In another embodiment of the invention, the antisense oligonucleotide comprises at least eight contiguous
20 nucleotides derived from the sequence set forth in SEQ ID NO:10.

In still another embodiment of the invention, the antisense oligonucleotide has the nucleotide sequence set forth in SEQ ID NO:14. In
25 another embodiment of the invention, the antisense oligonucleotide comprises at least eight contiguous nucleotides derived from the sequence set forth in SEQ ID NO:14.

30 The present invention also provides a method of inhibiting the activation of PKR in a cell culture.

10

The method comprises contacting said cell culture with
an effective amount of at least one of an isolated
1 nucleic acid molecule complementary to SEQ ID NO:1, an
isolated nucleic acid molecule complementary to
nucleotides 178-430 of SEQ ID NO:1, an isolated
nucleic acid molecule complementary to nucleotides
5 263-283 of SEQ ID NO:1, an isolated nucleic acid
molecule complementary to nucleotides 374-393 of SEQ
ID NO:1; an oligonucleotide having the sequence set
forth in SEQ ID NO:6 or at least eight contiguous
10 nucleotides of SEQ ID NO:6, an oligonucleotide having
the sequence set forth in SEQ ID NO:7 or at least
eight contiguous nucleotides of SEQ ID NO:7, an
oligonucleotide having the sequence set forth in SEQ
ID NO:10 or at least eight contiguous nucleotides of
15 SEQ ID NO:10, or an oligonucleotide having the
sequence set forth in SEQ ID NO:14 or at least eight
contiguous nucleotides of SEQ ID NO: 14.

In another aspect of the invention,
compositions for stimulating cell proliferation
(expansion) in cell cultures are provided. The
20 compositions comprise at least one of the subject
isolated nucleic acid molecules or antisense
oligonucleotides admixed with a pharmaceutically
acceptable carrier.

In another aspect of the present invention,
25 kits are provided which comprise at least one of the
subject isolated nucleic acid molecules or antisense
oligonucleotides and a pharmaceutically acceptable
carrier. The pharmaceutical acceptable carrier may be

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packaged separately or admixed with the subject
isolated nucleic acid molecules or oligonucleotides.

1 A method of stimulating cell proliferation
in a cell culture by contacting the cell culture with
an effective amount of at least one of the subject
isolated nucleic acid molecules or oligonucleotides is
5 also provided. For example, cells which are typically
difficult to grow and regenerate such as nerve, muscle
or brain cells, can be expanded by such a method. In
addition, epithelial cells can be expanded by
10 contacting cultured cells with an effective amount of
at least one of the subject antisense fragments or
oligonucleotides prior to using the cultured cells in
a skin grafting procedure.

A method of inhibiting cell proliferation in
bone marrow cells obtained from a patient suffering
15 from a hematological cancer is also provided by the
present invention. The method comprises isolating a
bone marrow sample from a patient suffering from a
hematological cancer, contacting the cells in said
sample with at least one of an R-RNA having the
20 sequence set forth in SEQ ID NO:2, a portion of an R-
RNA having nucleotides 178-430 of SEQ ID NO:2, a
portion of an R-RNA having nucleotides 263-283 of SEQ
ID NO:2, or a portion of an R-RNA having nucleotides
374-393 of SEQ ID NO:2, and after a sufficient time,
25 transplanting said sample back into the donor patient.

In another aspect of the invention, a method for
promoting expansion of pluripotent progenitor cells is
provided. The method comprises obtain bone marrow
30 cells from a patient and contacting said cells with an

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effective amount of an oligonucleotide having the nucleotide sequence set forth in SEQ ID NO:10.

1 The present invention further provides a method for promoting expansion of hematopoietic stem cells in a cell culture which comprises obtaining peripheral blood from a patient, isolating mononuclear
5 cells and contacting said mononuclear cells with an effective amount of an oligonucleotide having the nucleotide sequence set forth in SEQ ID NO:10.

 In a further aspect of the invention, a method for promoting neutrophil expansion and
10 development from a neutrophil depleted marrow cell culture is provided. In one embodiment, marrow from a patient suffering from severe congenital neutropenia (SCN) is obtained and contacted with an effective
15 amount of an oligonucleotide having the sequence set forth in SEQ ID NO:10. After a sufficient time, the marrow cells are transplanted back into the patient.

 A method for expanding hematopoietic cells in umbilical cord blood is also provided. The method
20 comprises contacting a sample of the cord blood with at least one oligonucleotide selected from the group consisting of an oligonucleotide having the sequence set forth in SEQ ID NO:8, an oligonucleotide having at
25 least eight contiguous nucleotides as set forth in SEQ ID NO:8, an oligonucleotide having the sequence set forth in SEQ ID NO:10 or an oligonucleotide having at least eight contiguous nucleotides as set forth in SEQ ID NO:10.

 The present invention further provides
30 pharmaceutical compositions comprising the subject

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nucleic acid fragments and oligonucleotides. Such pharmaceutical compositions are useful in practicing the methods of the present invention. Kits which comprise at least one of the subject isolated nucleic acid molecules or oligonucleotides and a pharmaceutically acceptable carrier are also provided.

Figure 1A is an autoradiogram which depicts the effect of the R-RNA transcript on PKR phosphorylation. All RNA transcripts were gel purified and added to protein kinase assays containing 3T3-F442A S10 extracts or purified 3T3-PKR to give the final concentrations indicated. The reaction mixtures contained the following additions: Lane 1, no RNA; Lane 2, 150 ng/ml poly(I)•poly(C) (PIC); Lanes 3,5,7,9 and 11, 5.0 µg/ml RNA from clones R-12, R-18, R-33, R-15 and R101 as indicated; Lanes 4,6,8,10 and 12, 0.50µg/ml RNA from clones indicated. The migration position and molecular weights ($\times 10^3$) of protein standards, phosphorylase (94 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa) are indicated on the left. The arrow indicates the position of PKR.

Figure 1B is an autoradiogram depicting the effect of the R-RNA transcript on PKR phosphorylation under the same conditions as depicted in Figure 1A. Lane 1, no RNA; Lane 2, 150 ng/ml poly(I)•poly(C) (PIC); Lanes 3-5, 2.5 µg/ml (Lane 3), 1.25 µg/ml (Lane 4) and 625 ng/ml (Lane 5) of the globin RNA transcript (G-Txpt); Lanes 6-8, 2.5 µg/ml (Lane 6) 1.25 µg/ml (Lane 7) and 625 ng/ml (Lane 8) of the R-RNA transcript (R-Txpt). The migration position and

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molecular weights ($\times 10^3$) of protein standards are as in Fig. 1A. The arrow indicates the position of PKR.

1 Figure 2A is a schematic diagram of the 847 bp R-RNA cDNA and indicates the constructs prepared, the location of the Hind III sites and the size of the DNA fragments expected from both orientations. The
5 orientations have been designated as antisense orientation (i.e., complementary to the authentic R-RNA, the resulting transcript (Txpt) hybridizes to the A⁺-RNA) and sense orientation.

10 Figure 2B is a photograph of an ethidium bromide stained agarose gel after electrophoresis of Hind III digested pGEM3Zf(+). Lane 1 contains Hae III digested Phi X 174 DNA as a marker. Lane 2 contains a 337 bp fragment as expected for the construct in the antisense orientation. Lane 3
15 contains a 612 bp fragment as expected for the construct in the sense orientation.

Figure 2C is an autoradiogram of a dot blot where poly(A)⁺ and (A)⁻-RNA prepared from confluent cultures of 3T3-F442A cells were applied to two
20 separate nitrocellulose sheets in the amounts indicated followed by separate hybridization with the ³²P-labeled antisense or sense transcript.

Figure 3A depicts the nucleotide sequence (SEQ ID NO:1) and alignment of the isolated R-RNA cDNA
25 fragment. The deduced amino acid sequence is shown below the nucleotide sequence. The amino acid sequence shown underlined was used to generate antiserum.

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Figure 3B is an autoradiogram of a Western blot using immune serum (1:200). Cell extracts containing 20 μ g of protein were applied to each lane as follows: Lane 1, 3T3-F442A S10 extract; Lane 2, NIH-3T3 S10 extract; Lane 3, *E. coli* DH-5 α extract; Lane 4, MG63 human osteosarcoma S10 extract; Lane 5, rabbit reticulocyte lysate (Promega). The migration position of several kaleidoscope prestained standard proteins (BIO-RAD) are indicated. Arrows indicate position of a non-specific bacterial protein antigenic to both immune and pre-immune sera. The arrowhead indicates the position of the 62 KDa protein specifically reading with the anti-peptide serum.

Figure 3C is an autoradiogram of a Western blot using preimmune serum (1:200) with protein applied as described for Figure 3B. The migration position of several kaleidoscope prestained standard proteins (BIO-RAD) are indicated. Arrows indicate position of a non-specific bacterial protein antigenic to both immune and pre-immune sera. The arrowhead indicates the position of the 62 KDa protein.

Figure 4A is an ethidium bromide stained electrophoretic gel depicting the distribution of the R-RNA DNA sequence. PCR amplified template genomic DNA (1 μ g), from mouse 3T3-F442A, mouse liver tissue, human CEM, and yeast cells were loaded as indicated. The migration position of several fragments of Hind III digested lambda DNA is shown on the left.

Figure 4B is an autoradiogram of a southern blot. The agarose gel was loaded with EcoRI digested genomic DNA (30 μ g) prepared from human CEM cells,

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sheep liver tissue, rat liver tissue, mouse 3T3-F442A cells, mouse liver tissue, and from yeast as indicated. The blot was hybridized to ^{32}P -labeled R-RNA partial cDNA (1.3×10^9 cpm/ μg , 1×10^7 cpm/ml) and exposed for two weeks at -76°C with an intensifying screen. The approximate size of the hybridizing bands is shown on the left and was determined from the migration position of Hae III digested Phi X 174 DNA.

Figure 5A is a Northern blot analysis of the R-RNA. Cellular RNAs were separated on a 1% formaldehyde denaturing agarose gel and transferred to nitrocellulose paper. The blot was hybridized to ^{32}P -labeled R-RNA partial cDNA (4.5×10^8 cpm/ μg , 1×10^6 cpm/ml of hybridization buffer). The gel was loaded as follows: Lane 1, 30 μg of human CEM cell poly (A)⁺ RNA (Human A⁺); Lane 2, 30 μg of 3T3-F442A cell poly(A)⁺RNA (3T3 A⁺) and Lane 3, 30 μg of 3T3-F442A cell-poly (A)⁻ RNA (3T3 A⁻).

Figure 5B is a Northern blot analysis of total cytoplasmic RNA (40 μg) prepared from 3T3-F442A cells. The probes used for hybridization were as follows: Lane 1, 0.7 μg R-RNA cDNA probe (1×10^8 cpm/ μg), Lane 2, 0.3 μg actin DNA probe (0.7 Kb, 1×10^8 cpm/ μg).

Figure 6 is an autoradiogram of a southern blot which demonstrates the effect of the R-RNA cDNA on R-RNA activity. DNA/RNA hybridization reactions (10 μl) containing the R-RNA transcript (20 ng) (Txpt), the isolated R-RNA (100 ng) and the total cytoplasmic RNA fraction (2 μg) (Cyt) were carried out

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in the presence and absence of 5-fold excess gel
purified R-RNA cDNA . Aliquots (5 μ l) were added to
protein kinase assays containing 3T3-F442A cell
extract as indicated in the figure. Other additions
contained hybridization buffer (Lane 1); hybridization
buffer and 150 ng/ml poly(I)•poly(C) (PIC) (Lane 2)
and hybridization buffer and 250 μ g/ml R-RNA cDNA
(Lane 9).

Figure 7 is an autoradiogram of an agarose
gel run with samples of the protein kinase assay with
added R-RNA transcript. High levels of
poly(I)•poly(C) (50 μ g/ml) were added to some assays
as indicated prior to incubation. Assays contained
the following additions. Lane 1, no RNA; Lane 2 and
3, 150 ng/ml poly(I)•poly(C) (PIC); Lanes 4-9
contained R-RNA transcript (Txpt) that has been
treated as follows: Lane 4 and 5, no treatment; Lanes
6, RNase T1 (T); Lane 7, RNase V1 (V), Lane 8, 100 C
for 2 min and slow cooled (Δ); Lane 9, 100 for 2 min
and rapidly cooled (Δ -C); High concentrations of
poly(I)•poly(C) (H•PIC) were added to assay indicated
in Lanes 3 and 5.

Figure 8 is a photograph of an ethidium
bromide stained gel after electrophoresis of PCR
reactions and demonstrates the association of the
R-RNA with PKR. Total cytoplasmic RNA (Cyt RNA, 2
 μ g); poly (A)⁺ RNA (A⁺ RNA, 0.4 μ g); poly (A)⁻ RNA (A⁻,
RNA, 0.4 μ g) and extracted RNA (R-RNA, 0.3 μ g) were
added to reactions as indicated. Control assays
containing no added RNA (-RNA) or no oligo (dT) primer
(R-RNA - oligo (dT)) were performed as indicated. In

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one assay direct PCR was carried out using the R-RNA cDNA (cDNA, 1.0 ng) as a template. The migration position of several fragments of Hpa I digested T7 DNA is shown on the left.

Figure 9 graphically depicts a comparison between Poly(I)-Poly(C) and R-RNA in the autophosphorylation of PKR. Poly(I)-poly(C) and the R-RNA transcript were added to protein kinase assays at the final concentrations indicated in the figure. The extent of phosphorylation of PKR was determined by scanning densitometry (OD 500 nm) of autoradiograms. Solid diamonds represent Poly(I)-poly(C). Open squares represent R-RNA.

Figure 10A is an autoradiogram demonstrating the Effect of RNase Digestion on the Activity of the R-RNA Transcript. After radiolabeled or unlabeled R-RNA transcript was digested with either RNase T1 or RNase VI, digestion reactions using radiolabeled transcript were subjected to electrophoresis on 5% polyacrylamide gels. Lane 1, Radiolabeled pGEM-3zf(-) digested with Hpa II; lane 2, R-RNA transcript without RNase digestion; lane 3-6, digestion with RNase T1 at a final activity of 0.78, 1.56, 3.13, and 12.5 units/ml respectively; lanes 7-11, digestion with RNase VI at a final activity of 0.05, 0.10, 0.19, 0.39 and 0.78 units/ml respectively. The figure is an autoradiogram.

Figure 10B is an autoradiogram of a gel loaded with the digestion reactions of Fig. 10A added to protein kinase containing purified PKR. Lane 1, no added RNA; lane 2, 150 ng/ml poly(I)-poly(C); lanes 3-

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7, RNase T1 digestion reactions (2-6); lanes 8-12, RNase VI digestion reactions (7-11). The final activity of the RNases in the digestion reactions was the same as in Fig. 10A.

Fig. 11A is a schematic diagram indicating the Alu I restriction map of the 847 bp R-RNA cDNA. Five Alu I fragments were predicted from the sequence.

Figure 11B is a photograph of an ethidium bromide stained agarose gel through which were electrophoresed Alu I fragments obtained from the digestion of the 847 bp R-RNA cDNA. The 26 bp fragment is not shown. Lane 1, a 480 bp DNA marker; lane 2, Alu 284 bp; lane 3, Alu 226 bp; lane 4, Alu 178 bp; lane 5, Alu 133 bp. Migration position of DNA of known size ladder is shown on far left lane.

Figure 11C is an autoradiogram demonstrating the effect of Alu I Fragments on the Phosphorylation of PKR. The gel was loaded with hybridization reactions containing purified R-RNA transcript and 10-fold molar excess of Alu I cDNA fragments. Additions of the different Alu I fragments are indicated by a "+". As controls for PKR, one assay contained no added RNA (lane 1) while another assay was supplemented with poly(I)-poly(C) (150 ng/ml) (lane 2) in the kinase reaction. The migration position and molecular weights ($\times 10^3$) of protein standards, phosphorylase (94 KDa), bovine serum albumin (67 KDa), ovalbumin (45 KDa) and carbonic anhydrase (30 KDa) are indicated on left. Arrow on right indicates the position of PKR.

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Figure 11D is a photograph of an ethidium bromide stained agarose gel through which were electrophoresed reactions containing gel purified R-RNA transcript incubated in the presence of 10-fold molar excess of Alu I-284 fragment (lane 2) and Alu I-226 fragment (lane 3). One assay contained only R-RNA transcript (lane 1).

Figure 12A is a schematic diagram indicating the location and spatial distribution of 11 oligomers complementary to the 252 nt region of the R-RNA. Also indicated are the gaps and overlaps in nucleotides between oligos. The numbers in the lower portion of the figure represent the position of the 226 activation sequence within the R-RNA cDNA sequence.

Fig. 12B is an autoradiogram which demonstrates the effect of the subject oligomers on the phosphorylation of PKR. Hybridization reactions and protein kinase assays were carried out as described in Example 10 for Fig. 11C, except that oligomers (50 ng) were added to hybridization reactions as indicated. Controls included hybridization reactions containing: no added RNA (lane 1), poly(I)-poly(C) (lane 2), and R-RNA transcript alone (lane 3).

Figure 12C is an autoradiogram depicting the effect of the subject oligomers on the phosphorylation of PKR by Poly (I)-Poly (C). Hybridization reactions and protein kinase assays were carried out as described in Example 10 for Fig. 3C except poly (I)-poly(C) (150 ng/ml) and oligomers (100 ng) were added

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to reactions as shown. One hybridization contained no added RNA.

1 Figure 13 is a photograph of an ethidium
bromide stained agarose gel through which was
electrophoresed reactions containing the gel purified
R-RNA transcript incubated in the presence of the
5 indicated complementary oligomer (200 ng). Stability
of the R-RNA was not effected by the oligomers.

Figure 14 graphically depicts the effect of
antisense oligomers OL-1, OL-2, OL-3, and OL-4 on cell
growth. ODN refers to oligodeoxynucleotide (the
10 antisense oligomers) which were added on day 6. Values
at each time point are the average of two independent
experiments each determined in duplicate. Solid
circles represent medium alone; open circles represent
an unrelated oligonucleotide; open triangles represent
15 OL-1; solid triangles represent OL-2; open squares
represent OL-3; solid squares represent OL-4; solid
diamonds represent a mixture of an equal amount of
each of OL-1, OL-2, OL-3, and OL-4.

Figure 15 graphically depicts the effects of
20 OL-1, OL-2, OL-3, and OL-4 on the expansion of burst
forming units of erythroid lineage (BFU-E). Human
peripheral blood mononuclear cells were treated with 1
 μ M of oligomers OL-1, OL-2, OL-3, and OL-4. Solid bar
represents cells in medium alone, open bar represents
25 treatment with unrelated oligomer.

Figure 16 graphically depicts the effect of
OL-2 on expansion of BFU-E at the different
concentrations indicated.

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Figure 17 graphically depicts the effect of OL-2 at a concentration of 1 μ M on expansion of BFU-E at the different time points indicated.

The present invention provides both a regulatory RNA (R-RNA) and a partial cDNA corresponding to the R-RNA. The R-RNA interacts with and stimulates autophosphorylation of PKR. In accordance with the present invention, the R-RNA may be used in different cell cultures in order to inhibit cell proliferation. For example, inhibition of cancerous cells in a bone marrow cell culture may be obtained by contacting a bone marrow cell culture isolated from a patient suffering from a hematological cancer with the R-RNA of the present invention. The nucleotide sequence of the subject R-RNA is set forth in SEQ ID NO:2.

Also in accordance with the present invention, a portion of the R-RNA responsible for activation of PKR has been identified. The activation sequence is approximately 226-252 nt in length and is made up of nucleotides 178-404 and can include an additional 26 nucleotides for a fragment comprising nucleotides 178-430 of SEQ ID NO:2. Thus, the R-RNA activation sequence alone may be used in a method of inhibiting cell proliferation in different cell cultures.

The present invention also provides a partial cDNA corresponding to the R-RNA. The cDNA sequence is approximately 847 nt long and has the sequence set forth in SEQ ID NO:1. In addition, sequence corresponding to the R-RNA activation

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sequence located at nucleotides 178-404 or 178-430 of
SEQ ID NO:1 is also provided. Sequences complementary
1 to the sense strand of the R-RNA cDNA, including the
activation sequence or portions thereof, are useful in
methods of inhibiting the activation of PKR and
5 promoting cell expansion in cell cultures.

For example, an isolated nucleic acid
fragment having either the nucleotide sequence as set
forth in SEQ ID NOS:1 or 2, or nucleotides 178-404 or
178-430 of SEQ ID NOS:1 or 2 or having at least a 50%
similarity thereto is useful in methods of inhibiting
10 cell expansion in a cell culture. An isolated nucleic
acid fragment complementary to the nucleotide sequence
as set forth in SEQ ID NOS:1 or 2 or having at least a
50% complementarity is useful in promoting cell
15 proliferation in cell cultures.

A nucleotide sequence which is complementary
to the 226-252 nt fragment corresponding to
nucleotides 178-404 or 178-430 of SEQ ID NOS:1 or 2 or
having at least a 50% complementarity thereto is a
20 preferred fragment for inhibiting the activation of
PKR and promoting cell proliferation in cell cultures.
Other preferred fragments include a nucleotide
sequence which is complementary to nucleotides 263-283
of SEQ ID NOS:1 or 2 or having at least a 50%
25 complementarity thereto, and a nucleotide sequence
which is complementary to nucleotides 374-393 of SEQ
ID NOS:1 or 2 or having at least a 50% complementarity
thereto.

Other nucleotide sequences which hybridize
30 to portions of the activation sequence of the subject

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R-RNA are also contemplated by the present invention. For example, isolated nucleic acid molecules which
1 hybridize to nucleotides 178-430 of SEQ ID NO:2 under medium to high stringency conditions are contemplated as are isolated nucleic acid molecules which hybridize
5 to nucleotides 263-283 of SEQ ID NO:2 under medium to high stringency conditions. Also contemplated by the present invention are isolated nucleic acid molecules which hybridize to nucleotides 374-393 of SEQ ID NO:2 under medium to high stringency conditions. Such
10 isolated nucleic acid molecules are useful in promoting cell proliferation when used in conjunction with cell culture methods.

Preferred percentage similarities or complementarity include 80%, 85%, 90%, 92-95%, 96-98% and 99-100%. However, nucleic acid molecules having
15 at least 50% similarities to SEQ ID NO:1 and portions thereof as defined herein and which can be used in various reverse transcription reactions in order to produce an R-RNA which activates PKR and inhibits cell proliferation are within the scope of the present
20 invention. Similarly, nucleic acid molecules having at least 50% complementarities to SEQ ID NO:1 and portions thereof as defined herein and which inhibit the activation of PKR and stimulate cell proliferation are within the scope of the present invention.

25 As used herein, hybridization under medium or high stringency conditions are as defined in Maniatis et al. 1982 *Molecular Cloning*, Cold Spring Harbor Laboratory, N.Y., at pages 387-389, and
30 especially paragraph 11 which is herein incorporated

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by reference. A low stringency is defined as being in 4-6X SSC/1% (w/v) SDS at 37-45°C for 2-3 hours.

1 Medium stringency conditions are considered herein to be 1-4X SSC/0.5%-1% (w/v) SDS at greater than or equal to 45°C for 2-3 hours. High stringency conditions are
5 considered herein to be 0.1-1X SSC/0.1-1% (w/v) SDS at greater than or equal to 60°C for 1-3 hours. As used herein, medium to high stringency conditions refer to conditions which are either medium stringency conditions, high stringency conditions, or conditions
10 between medium and high stringency.

15 A cDNA corresponding to R-RNA can be provided by first fractionating poly(A)⁺ by cellulose chromatography followed by cDNA synthesis using any number of widely known methods and commercially available kits. Identification of a cDNA
20 corresponding to an R-RNA and isolation of the R-RNA may be achieved by protein kinase assays where R-RNA transcribed from an isolated cDNA clone placed under the control of a promoter in an expression vector, facilitates phosphorylation of the 67 KDa
25 phosphoprotein known as PKR. Protein kinase assay methodologies are widely known and can be found e.g., in Petryshyn et al., 1983 *Methods Enzymol.* 9:346-362 and Petryshyn et al., 1988 *Proc. Natl. Acad. Sci. U.S.A.* 85:1427-1431. Specific fragments of the R-RNA
30 cDNA may be isolated using various restriction enzymes which are widely available. Such enzymes have known restriction sites which can be mapped to the R-RNA cDNA. For example, the R-RNA cDNA may be restricted with HindIII to render a 337 bp and 612 bp fragment

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(Fig. 2A). It may also be used to render fragments of 284, 226, 178, 133 and 26 base pairs (Fig. 11A).

1 The present invention also provides
oligonucleotides which are useful for inhibiting the
activation of PKR and stimulating cell proliferation
under cell culture conditions. The subject
5 oligonucleotides of the present invention comprise at
least eight or nine nucleotides which are
complementary to at least eight or nine contiguous
nucleotides of nucleotides 178-430 as set forth in SEQ
ID NOs:1 or 2. Other antisense oligonucleotides
10 include oligonucleotides comprising at least eight or
nine nucleotides which are complementary to: at least
eight or nine contiguous nucleotides of nucleotides
263-283 as set forth in SEQ ID NOs:1 or 2, or at least
eight or nine contiguous nucleotides of nucleotides
15 374-393 as set forth in SEQ ID NOs: 1 or 2. Larger
antisense oligonucleotides of, for example, 10 to
greater than 25 nucleotides are also contemplated by
the present invention. The skilled artisan is
20 cognizant of the many different methods in which to
make such oligonucleotides and methods for testing
whether such oligonucleotides have the capacity to
inhibit activation of PKR and stimulate cell
proliferation under cell culture conditions. Methods
25 for testing whether oligonucleotides inhibit the
activation of PKR and stimulate cell proliferation are
provided in the working examples e.g., protein kinase
assays and cell culture studies.

30 The lower limit to the length of a subject
oligonucleotide (eight or nine nucleotides) is based

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1 upon well known principles of deoxyribonucleotide and
ribonucleotide binding. As is known by those of skill
in the art, usually at least eight or nine nucleotides
are necessary to provide stable binding among
ribonucleotide and deoxyribonucleotide sequences.

5 Since the methods of the present invention involve use
of the subject oligonucleotides in order to bind to R-
RNA, a lower limit of eight or nine nucleotides is
contemplated for the subject oligonucleotides.

The present invention also provides specific
oligonucleotides which comprise nucleotides from the
10 antisense strand of SEQ ID NOs:1 or 2. For example,
an oligonucleotide may comprises the sequence set
forth in SEQ ID NO:6 or at least eight contiguous
oligonucleotides derived from the sequence set forth
in SEQ ID NO:6. Other oligonucleotides provided by
15 the present invention include an oligonucleotide
comprising the sequence set forth in SEQ ID NO:7 or at
least eight contiguous oligonucleotides derived from
the sequence set forth in SEQ ID NO:7, an
oligonucleotide comprising the sequence set forth in
20 SEQ ID NO:8 or at least eight contiguous
oligonucleotides derived from the sequence set forth
in SEQ ID NO:8, and an oligonucleotide comprising the
sequence set forth in SEQ ID NO:14 or at least eight
contiguous oligonucleotides derived from the sequence
25 set forth in SEQ ID NO:14.

In a preferred embodiment, the
oligonucleotide comprises the sequence set forth in
SEQ ID NO:8 or at least eight contiguous
oligonucleotides derived from the sequence set forth
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in SEQ ID NO:8, In another preferred embodiment, the
oligonucleotide comprises the sequence set forth in
1 SEQ ID NO:10 or at least eight contiguous
oligonucleotides derived from the sequence set forth
in SEQ ID NO:10.

5 Modifications to the oligonucleotides set
forth in SEQ ID NOs:6, 7,8, 10 and 14 which bind to R-
RNA and which maintain the characteristic property of
interfering with the interaction of R-RNA to PKR are
also within the scope of the present invention. Such
10 modifications include insertions, deletions and
substitutions of one or more nucleotides.

The isolated nucleic acid fragments and
oligonucleotides of the present invention are DNA or
RNA, or hybrids of DNA and RNA. For example, the
subject nucleic acid fragments and oligonucleotides
15 can comprise: all RNA; all DNA; RNA interspersed with
2'-O-methyl RNA, and so on. The nucleic acid
fragments and oligonucleotides comprise the bases
guanine (G), adenine (A), thymine (T), cytosine (C) or
uracil (U) in the nucleotides, or any nucleotide
20 analog that is capable of binding to R-RNA.
Nucleotide analogs include pseudocytidine,
isopseudocytidine, imidazole, 3-aminophenyl-imidazole,
2'-O-methyl-adenosine, 7-deazadenosine, 7-
deazaguanosine, 7-deazaxanthosine, 4-acetylcytidine,
25 5-(carboxy-hydroxymethyl)-uridine, 2'-O-
methylcytidine, 5-carboxymethylaminomethyl-2-
thioridine, 5-carboxymethylamino-methyluridine,
dihydrouridine, 2'-O-methyluridine, pseudouridine, 2'-
O-methyl-pseudouridine, beta, D-galactosylqueosine,
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2'-O-methylguanosine, inosine, N6-
 1 isopentenyladenosine, 1-methyladenosine, 1-methyl-
 pseudouridine, 1-methylguanosine, 1-methylinosine,
 2,2-dimethylguanosine, 2-methyladenosine, 2-
 methylguanosine, 3-methylcytidine, 5-methylcytidine,
 5-methyluridine, N6-methyl-adenosine, 7-
 5 methylguanosine, 5-methylamino-methyluridine, 5-
 methoxyaminomethyl-2-thiouridine, β -D-
 mannosylqueosine, 5-methoxycarbonylmethyluridine, 5-
 methoxyuridine, 2-methyl-thio-N6-isopentenyladenosine,
 10 N-(9-beta-D-ribofuranosyl-2-methylthiopurine-6-yl)-
 carbamoyl)threonine, N-(9-beta-D-ribofuranosylpurine-
 6-yl)-N-methylcarbamoyl)threonine and thioguanosine.

Either ribose or deoxyribose sugars can be
 used with the above-listed analogs. Modified sugars,
 such as 2'-O-methyl ribose, are also contemplated.
 15 Nucleotides bases in an α -anomeric conformation can
 also be used in the isolated nucleic acids and
 oligonucleotides of the present invention.

Preferred nucleotides analogs are unmodified
 G, A, T, C and U nucleotides; pyrimidine analogs with
 20 lower alkyl, alkynyl or alkenyl groups in the 5
 position of the base and purine analogs with similar
 groups in the 7 or 8 position of the base. Especially
 preferred nucleotide analogs are 5-methylcytosine, 5-
 methyluracil, diaminopurine, and nucleotides with a
 25 2'-O-methylribose moiety in place of ribose or
 deoxyribose. As used herein lower alkyl, lower
 alkynyl and lower alkenyl contain from 1 to 6 carbon
 atoms and can be straight chain or branched. These
 30 groups include methyl, ethyl, propyl, isopropyl,

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butyl, isobutyl, tertiary butyl, amyl, hexyl and the like. A preferred alkyl group is methyl.

1 The isolated nucleic acid molecules of the
present invention may be generated from larger nucleic
acid fragments having excess sequence on either or
both 3' and 5' ends removed via exonuclease III-
5 mediated deletion. This is accomplished by digesting
appropriately prepared DNA with exonuclease III
(exoIII) and removing aliquots at increasing intervals
of time during the digestion. The resulting
successively smaller fragments of DNA may be sequenced
10 to determine the exact endpoint of the deletions.
There are several commercially available systems which
use exonuclease III (exoIII) to create such a deletion
series, e.g. Promega Biotech, "Erase-A-Base" system.
Alternatively, PCR primers can be defined to allow
15 direct amplification of the subject nucleic acid
fragments.

The subject nucleic acid molecules and
oligonucleotides of the present invention can also be
made by any of a myriad of procedures known for making
20 DNA or RNA oligonucleotides. For example, such
procedures include enzymatic synthesis and chemical
synthesis.

Enzymatic methods of DNA oligonucleotides
synthesis frequently employ Klenow, T7, T4, Tag or E.
25 coli DNA polymerase as described in Sambrook et al.
1989, in *Molecular Cloning: A Laboratory Manual*, Cold
Spring Harbor, NY. Enzymatic methods of RNA
oligonucleotide synthesis frequently employ SP6, T3 or
30 T7 RNA polymerase as described in Sambrook et al.,

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1989. Reverse transcriptase can also be used to
synthesize DNA from RNA (Sambrook et al. 1989). To
1 prepare oligonucleotides enzymatically requires a
template nucleic acid which can either be synthesized
chemically, or be obtained as mRNA, genomic DNA,
cloned genomic DNA, cloned cDNA or other recombinant
5 DNA. Some enzymatic methods of DNA oligonucleotide
synthesis can require an additional primer
oligonucleotide which can be synthesized chemically.
Finally linear oligonucleotides can be prepared by
polymerase chain reaction (PCR) techniques as
10 described, for example, by Saiki et al., 1988, *Science*
239: 487.

Chemical synthesis of linear
oligonucleotides is well known in the art and can be
achieved by solution or solid phase techniques.
15 Moreover, linear oligonucleotides of defined sequence
can be purchased commercially or can be made by any of
several different synthetic procedures including the
phosphoramidite, phosphite triester, H-phosphonate and
phosphotriester methods, typically by automated
20 synthesis methods. The synthesis method selected can
depend on the length of the desired oligonucleotide
and such choice is within the skill of the ordinary
artisan. For example, the phosphoramidite and
phosphite triester method produce oligonucleotides
25 having 175 or more nucleotides while the H-phosphonate
method works well for oligonucleotides of less than
100 nucleotides. If modified bases are incorporated
into the oligonucleotide, and particularly if modified
phosphodiester linkages are used, then the synthetic
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procedures are altered as needed according to known procedures. In this regard, Uhlmann, et al. (1990, *Chemical Reviews* 90: 543-584) provide references and outline procedures for making oligonucleotides with modified bases and modified phosphodiester linkages.

Synthetic linear oligonucleotides may be purified by polyacrylamide gel electrophoresis, or by any of a number of chromatographic methods, including gel chromatography and high pressure liquid chromatography. To confirm a nucleotide sequence, oligonucleotides may be subjected to DNA sequencing by any of the known procedures, including Maxam and Gilbert sequencing, Sanger sequencing, capillary electrophoresis sequencing, the wandering spot sequencing procedure or by using selective chemical degradation of oligonucleotides bound to Hybond paper. Sequences of short oligonucleotides can also be analyzed by laser desorption mass spectroscopy or by fast atom bombardment (McNeal, et al., 1982, *J. Am. Chem. Soc.* 104: 976; Viari, et al., 1987, *Biomed. Environ. Mass Spectrom.* 14: 83; Grotjahn et al., 1982, *Nuc. Acid Res.* 10: 4671). Sequencing methods are also available for RNA oligonucleotides.

The present invention also contemplates derivatization or chemical modification of the subject nucleic acid fragments and oligonucleotides with chemical groups to facilitate cellular uptake. For example, covalent linkage of a cholesterol moiety to an oligonucleotide can improve cellular uptake by 5- to 10- fold which in turn improves DNA binding by about 10- fold (Boutorin et al., 1989, *FEBS Letters*

254: 129-132). Other ligands for cellular receptors
may also have utility for improving cellular uptake,
1 including, e.g. insulin, transferrin and others.
Similarly, derivatization of oligonucleotides with
poly-L-lysine can aid oligonucleotide uptake by cells
(Schell, 1974, *Biochem. Biophys. Acta* 340: 323, and
5 Lemaitre, et al., 1987, *Proc. Natl. Acad. Sci. USA* 84:
648). Certain protein carriers can also facilitate
cellular uptake of oligonucleotides, including, for
example, serum albumin, nuclear proteins possessing
10 signals for transport to the nucleus, and viral or
bacterial proteins capable of cell membrane
penetration. Therefore, protein carriers are useful
when associated with or linked to the
oligonucleotides of this invention. Accordingly, the
present invention contemplates derivatization of the
15 subject oligonucleotides with groups capable of
facilitating cellular uptake, including hydrocarbons
and non-polar groups, cholesterol, poly-L-lysine and
proteins, as well as other aryl or steroid groups and
polycations having analogous beneficial effects, such
20 as phenyl or naphthyl groups, quinoline, anthracene or
phenanthracene groups, fatty acids, fatty alcohols and
sesquiterpenes, diterpenes and steroids.

Derivatization of the subject nucleic acid
fragments and oligonucleotides with groups that
25 facilitate cellular uptake or target binding, can be
done by any of the procedures known to one skilled in
the art. Moreover, the desired groups can be added to
nucleotides before synthesis of the oligonucleotide.
30 For example, these groups can be linked to the 5-

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position of T or C and these modified T and C
nucleotides can be used for synthesis of the present
oligonucleotides. In addition, derivatization of
selected nucleotides permits incorporation of the
group into selected domains of the subject
oligonucleotides.

In accordance with the present invention,
modification in the phosphodiester backbone of the
subject oligonucleotides is also contemplated. Such
modifications can aid uptake of a subject
oligonucleotide by cells or can extend the biological
half-life of such oligonucleotides. For example, the
subject oligonucleotides may penetrate the cell
membrane more readily if the negative charge on the
internucleotide phosphate is eliminated. This can be
done by replacing the negatively charged phosphate
oxygen with a methyl group, an amine or by changing
the phosphodiester linkage into a phosphotriester
linkage by addition of an alkyl group to the
negatively charged phosphate oxygen. Alternatively,
one or more of the phosphate atoms that are part of
the normal phosphodiester linkage can be replaced.
For example, NH-P, CH₂-P or S-P linkages can be
formed. Accordingly, the present invention
contemplates using methylphosphonates,
phosphorothioates, phosphorodithioates,
phosphotriesters and phosphorus-boron (Sood, et al.,
1990, *J. Am. Chem. Soc.* 112: 9000) linkages. The
phosphodiester group can be replaced with siloxane,
carbonate, acetamidate or thioether groups. These
modifications can also increase the resistance of the

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subject oligonucleotides to nucleases. Methods for
1 synthesis of oligonucleotides with modified
phosphodiester linkages are reviewed by Uhlmann. et
al.

Additionally, different nucleotide sugars
5 can be incorporated into the oligonucleotides of this
invention. Additional binding stability can be
provided by using 2'-O-methyl ribose in the present
oligonucleotides. Phosphoramidite chemistry can be
used to synthesize RNA oligonucleotides as described
10 (Reese, C.B. in *Nucleic Acids and Molecular Biology*;
Springer-Verlag: Berlin, 1989; Vol. 3, p. 164; and
Rao, et al., 1987, *Tetrahedron Lett.* 28: 4897).

The synthesis of RNA 2'-O-methyl-
oligoribonucleotides and DNA oligonucleotides differ
15 only slightly. RNA 2'-O-methyloligonucleotides can be
prepared with minor modifications of the amidite, H-
phosphonate or phosphotriester methods (Shibahara, et
al., 1987, *Nucleic Acids Res.* 15: 4403; Shibahara, et
al., 1989, *Nucleic Acids Res.* 17: 239; Anoue, et al.,
20 1987, *Nucleic Acids Res.* 15: 6131).

The present invention also provides methods
of inhibiting cell proliferation and inducing cell
death by increasing apoptosis in a cell culture. The
method comprises contacting the cultured cells with an
25 R-RNA having the sequence set forth in SEQ ID NO:2 or
the cDNA having the sequence set forth in SEQ ID NO:1.
Portions of the R-RNA as set forth in SEQ ID NO:2 may
also be used in the method. Such RNA fragments may be
obtained by *in vitro* transcribing the corresponding
30 portions of the cDNA. Preferred R-RNA fragments

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include those having nucleotides 178-430 of SEQ ID NO:2, nucleotides 263-283 of SEQ ID NO:2, and nucleotides 374-393 of SEQ ID NO:2. Preferred cDNA fragments include those having nucleotides 178-430 of SEQ ID NO:1, nucleotides 263-283 of SEQ ID NO:1, and nucleotides 374-393 of SEQ ID NO:1. Methods of inhibiting cell proliferation and inducing cell death in a cell culture using the subject R-RNA and cDNA molecules are useful for example, in purging populations of tumor cells *ex vivo*. For example, such methods may be used to inhibit cell proliferation and induce cell death in bone marrow cells obtained from a patient suffering from a hematological cancer. In this embodiment, bone marrow cells are obtained from a patient suffering from a hematological cancer, the cells are contacted with an effective amount of R-RNA, R-RNA fragment, cDNA, or cDNA fragment, and after a sufficient time to allow inhibition of cell proliferation and cell death, such cells are transplanted back into the donor patient.

A further aspect of this invention provides methods for inhibiting the activation of PKR and methods for stimulating cell proliferation (expansion) in a cell culture. A method for inhibiting the activation of PKR comprises contacting the cultured cells with an effective amount of at least one of an isolated nucleic acid molecule complementary to SEQ ID NOs:1 or 2, an isolated nucleic acid molecule complementary to nucleotides 178-430 of SEQ ID NOs:1 or 2, an isolated nucleic acid molecule complementary to nucleotides 263-283 of SEQ ID NOs:1 or 2, an

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isolated nucleic acid molecule complementary to
nucleotides 374-393 of SEQ ID NOs:1 or 2, an
1 oligonucleotide having the sequence set forth in SEQ
ID NO:6 or at least eight contiguous nucleotides of
SEQ ID NO:6, an oligonucleotide having the sequence
set forth in SEQ ID NO:7 or at least eight contiguous
5 nucleotides of SEQ ID NO:7, an oligonucleotide having
the sequence set forth in SEQ ID NO:8 or at least
eight contiguous nucleotides of SEQ ID NO:8, an
oligonucleotide having the sequence set forth in SEQ
ID NO:10 or at least eight contiguous nucleotides of
10 SEQ ID NO:10, and an oligonucleotide having the
sequence set forth in SEQ ID NO:14 or at least eight
contiguous nucleotides of SEQ ID NO:14.

In a preferred embodiment, a method of
stimulating cell proliferation (expansion) in a cell
15 culture comprises contacting cells of the culture with
an effective amount of at least one of an
oligonucleotide having the sequence set forth in SEQ
ID NO:8 or SEQ ID NO:10 or at least eight contiguous
nucleotides of SEQ ID NO:8 or SEQ ID NO:10.
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In another aspect of the invention a method
for promoting expansion of pluripotent progenitor
cells is provided. The method comprises obtaining
bone marrow cells from a patient and contacting said
cells with an effective amount of at least one of an
25 oligonucleotide having the sequence set forth in SEQ
ID NO:10 or at least eight contiguous oligonucleotides
of SEQ ID NO:10, or an oligonucleotide having the
sequence set forth in SEQ ID NO:8, or at least eight
contiguous oligonucleotides of SEQ ID NO:8.
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The present invention also provides a method
for promoting expansion of hematopoietic stem cells
1 which comprises obtaining peripheral blood from a
patient, isolating mononuclear cells and contacting
said mononuclear cells with an effective amount of an
oligonucleotide having the nucleotide sequence set
5 forth in SEQ ID NO:10 or at least eight contiguous
oligonucleotides of SEQ ID NO:10.

A method for promoting neutrophil
expansion and development from a neutrophil depleted
marrow cell culture is also provided. The method
10 comprises obtaining marrow cells from a patient
suffering from severe congenital neutropenia (SCN),
contacting said cells with an effective amount of an
oligonucleotide having the sequence set forth in SEQ
ID NO:10 or at least eight contiguous oligonucleotides
15 of SEQ ID NO:10, and after a sufficient time to allow
neutrophil expansion, transplanting the marrow cells
back into the patient.

In another aspect of the invention, there is
provided a method for expansion of cells involved in
20 wounds and burns. In this embodiment, an effective
amount of at least one of the subject antisense
fragments or oligonucleotides are applied directly to
a wound or burn on a subject. Alternatively, an
effective amount of at least one of the subject
25 antisense fragments or oligonucleotides are admixed
with a dressing and the dressing applied directly to
the wound or burn.

In still another aspect of the invention, a
method of expanding cells used in skin grafts is
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provided. The method comprises contacting epithelial
cells in culture with an effective amount of at least
one of the subject antisense fragments or
oligonucleotides prior to using the cultured cells in
a skin grafting procedure.

In yet another aspect of the present
invention, there is provided a method of promoting
growth and expansion of cells which are typically
difficult to grow and regenerate. Such cells include
nerve, muscle and brain cells. The method comprises
contacting a culture of nerve, muscle, brain or any
other cell type which is recalcitrant to growth and
regeneration, with an effective amount of at least one
of the subject antisense fragments or oligonucleotides
for a sufficient time so as to stimulate cell
expansion.

In another aspect of the invention, there is
provided a method for preventing cell death due to
apoptosis by contacting said cells with at least one
of the subject isolated antisense fragments and
oligonucleotides. Such a method finds particular use
in expansion and maintenance of hematopoietic cell
populations by minimizing programmed cell death.

In still another aspect of the present
invention, there is provided a method of expanding
hematopoietic cells in umbilical cord blood by
contacting a sample of the cord blood with at least
one of the subject antisense fragments or
oligonucleotides. Preferred oligonucleotides for
practicing this aspect of the invention include OL-1
(SEQ ID NO:8) and OL-2 (SEQ ID NO:10).

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As used herein, "cell culture" refers to any number of different vertebrate cell types which may be cultured according to methods well known to those skilled in the art. Preferred cell cultures are mammalian cell cultures. Preferred cell types for the cell cultures of the present invention include fibroblasts, bone marrow cells, mononuclear cells, neutrophils and hematopoietic stem cells. Hematopoietic stem cells include cells of erythroid lineage, granulocyte/macrophage lineage, or granulocyte/erythroid/myeloid/megakaryocyte lineage.

As used herein, "effective amount" refers to that amount which is effective in achieving the method. For example, in a method of inhibiting cell proliferation, an effective amount of R-RNA or portion of R-RNA as defined herein comprises that amount which is effective in inhibiting cell proliferation. In a method of inhibiting the activation of PKR or in stimulating cell proliferation (expansion), an effective amount of an isolated nucleic acid molecule complementary to SEQ ID NOs:1 or 2, portions thereof and oligonucleotides as defined herein, comprises that amount which is effective in inhibiting the activation of PKR or in stimulating cell proliferation. In particular, the subject oligonucleotides are added to cell cultures in an effective amount of about .10 μ M to 100 μ M final concentration, and preferably at about 1.0 to 10 μ M final concentration. Nucleic acid molecules (fragments) are added to cell cultures in an effective amount of about 1 ng to about 1000 ng/ml final

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concentration, and preferably at about 50 ng to about 150 ng/ml final concentration.

1 A further aspect of this invention provides pharmaceutical compositions containing the subject nucleic acid molecules and oligonucleotides and a
5 pharmaceutically acceptable carrier. Dosages can be readily determined by one of ordinary skill in the art based on preferred effective amounts and formulated into the subject pharmaceutical compositions.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion
10 media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are non-toxic to the particular cells in culture. The use of such media and agents for pharmaceutical active substances is well known in the art. Except
15 insofar as any conventional media or agent is incompatible with either the active ingredient, i.e., the subject nucleic acid fragments or oligonucleotides or the particular cell culture type, its use in the pharmaceutical compositions is
20 contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The present invention further provides kits which comprise at least one of the subject isolated nucleic acid molecules or oligonucleotides and a
25 pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may be packaged either separately from or admixed with the subject nucleic acid molecules and oligonucleotides.

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The invention is further illustrated by the
following specific examples which are not intended in
1 any way to limit the scope of the invention.

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EXAMPLE 11 ISOLATION AND ANALYSIS OF A PARTIAL
CDNA ENCODING R-RNA

5 In order to determine the number of RNAs
constituting the R-RNA activity, oligo(dT) primed cDNA
synthesis using isolated R-RNA fraction as a template
was carried out. Mouse 3T3-F442A cells were cultured
in Dulbecco-Vogt modification of Eagle's minimal
essential medium supplemented with 10% fetal calf
serum and the cell extracts were prepared as
10 previously described (Petryshyn, et al., 1984, *J.*
Biol. Chem. 259:14736-14742). Cytoplasmic RNA was
isolated from cultured 3T3-F442A and human CEM cells
and poly(A)⁺ and poly(A)⁻ RNA was separated by a
oligo[dT] cellulose column as previously described
15 (Li, et al., 1991, *Eur. J. Biochem.* 195:41-48). All
RNAs were dissolved in diethylpyrocarbonate treated
H₂O (0.1%) and used immediately or stored at -20°C.

R-RNA was isolated by fractionation of
poly(A)⁺ RNA on a CF-11 cellulose column as previously
20 described (Li, et al., 1991) and used as a template
for synthesis of cDNAs. cDNA synthesis was performed
using the cDNA Synthesis Plus System (Amersham)
according to manufacturer's instruction. RNAs were
heated at 95°C for 5 min and chilled on ice. Reverse
25 transcription reactions (20µl) contained 1 x Taq
polymerase buffer (Promega), 3.5mM MgCl₂, 1mM each
dNTP, 1 unit/µ RNasin, 100 pmol oligo (dT) primer and
7.5 units AMV reverse transcriptase. The mixtures
were preincubated for 10 min at 23°C, followed by
30 incubation for 60 min at 42°C. Reactions were

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terminated by heating at 95°C for 10 min. RNAs were
denatured with formaldehyde (Sambrook, et al., 1989,) before use. Three distinct cDNAs were visualized
after separation of reactions by electrophoresis in 1% agarose gels and autoradiography. The cDNAs were
methylated and EcoRI linkers were added to the cDNAs.
This procedure was followed by digestion with EcoRI as previously described (Sambrook, et al., 1989). The
modified cDNAs were ligated into the vector
pGEM-3Zf(-) at the β gal EcoRI site with T4 ligase.
The recombinant DNAs were then used to transform *E. coli* DH-5 α and recombinants were selected as white colonies on LB/Amp/X-gal plates. The recombinant DNAs
were isolated by standard procedure (Sambrook, et al., 1989) and linearized by digestion with Sac I. The
3'-overhang created by the Sac I digestion was removed
by treatment with Klenow fragment to reduce formation of artificial dsRNA during transcription (Schenborn, et al., 1985, *Nucl. Acids Res.* 13: 6223-6236). RNA
transcripts were made from the linearized recombinant DNA from the T7 RNA polymerase promoter using T7 RNA
polymerase (Promega) according to the manufacturer's recommended procedure. The α -globin RNA transcript
was prepared from the SP6 promoter site of plasmid pHST101 (Lee Gehrke, MIT) after digestion with Bam HI.
The transcripts were purified by electrophoresis on 1% agarose gels followed by electroelution of the RNA
band by using a unidirectional electroelutor (IBI). Each purified RNA transcript was added at several
concentrations to protein kinase assays containing

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latent PKR and the extent of kinase phosphorylation
was determined as described in Example 2.

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EXAMPLE 21 IDENTIFICATION OF R-RNA cDNA THROUGH
5 PROTEIN KINASE ASSAYS

The gel purified transcripts of the R-RNA cDNA were treated as follows before addition to the kinase assays. For RNase T1 treatment, transcript RNA (2 $\mu\text{g/ml}$) was incubated in a mixture containing 10 unit/ml RNase T1, 30 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM MgCl_2 , and 0.1 mM dithiothreitol for 15 min at 37°C. For RNase V1 treatment, transcript RNA (2 $\mu\text{g/ml}$) was incubated in a mixture containing 10 unit/ml RNase V1, 25 mM Tris-HCl (pH 7.2), 10 mM MgCl_2 , and 200 mM NaCl for 15 min at 37°C. For heat treatment, transcript RNA (2 $\mu\text{g/ml}$) was adjusted to 150 mM KCl and heated at 100°C for 2 min. The samples were either allowed to cool slowly to room temperature or were immediately frozen in a dry ice bath and thawed on ice. Aliquots (5 μl) were transferred to protein kinase assays to obtain a final concentration of 500 ng/ml transcript.

20 Protein kinase assays (20 μl) using 3T3-F442A extract (4-20 μg protein) or PKR purified from 3T3-F442A cells (0.25 μg) (Petryshyn, et al., 1983, *Methods Enzymol.* 9:346-362) were performed under conditions as described (Petryshyn, et al., 1984, *J. Biol. Chem.* 259: 14736-14742). Other additions are as indicated in Figures 1A and 1B. Proteins were separated by electrophoresis on 7.5% SDS-polyacrylamide gels and phosphoprotein profiles were analyzed following autoradiography (Ernst, et al., 1978, *J. Biol. Chem.* 253:7163-7172).

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Poly(I)•Poly(C) was obtained from Pharmacia and [γ - 32 P] ATP (4500 Ci/mmol) was obtained from ICN.

1 Although a substantial number of clones were
analyzed, only cDNA from one clone designated R-15
gave rise to an RNA transcript (~800 nucleotides)
which facilitated the phosphorylation of a 67 KDa
5 phosphoprotein previously identified as PKR
(Petryshyn, et al., 1994, *Progress in Molecular and
Subcellular Biology* 14:1-14; Petryshyn, et al., 1984,
J. Biol. Chem. 259:14736-14742). This cDNA was termed
R-RNA cDNA. The addition of this transcript to
10 protein kinase assays containing crude 3T3-cell
extract, (Fig. 1A, lanes 9 and 10) or highly purified
preparations of PKR (Fig. 1B, lanes 6-8), resulted in
a concentration dependent phosphorylation of PKR. In
contrast, no phosphorylation of the kinase was
15 observed in the absence of added RNA (Fig. 1A and B,
Lane 1). That the observed phosphorylation of PKR was
not due to either an adventitious RNA transcript or
was an artifact of *in vitro* transcription is
demonstrated by the finding that no phosphorylation of
20 PKR was observed with identical levels of similarly
prepared transcripts from all other randomly selected
clones tested (four of which are shown, Fig. 1B, lanes
3-8 and lanes 11-12), or upon addition of a similarly
prepared globin RNA transcript (Fig. 1B, Lanes 3-5).
25 Moreover, addition of the RNA transcript (269
nucleotides) obtained from the pGEM vector alone was
without effect.

 In addition, both sense and antisense RNA
probes to the R-RNA cDNA (SEQ ID NO:2 and the
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complementary sequence to SEQ ID NO:2, respectively)
were prepared and used to address the possibility that
1 the activation of PKR may be due to RNA:RNA hybrids
formed as a result of some opposite sense strand
synthesis during transcription (Schenborn, et al.,
1985, *Nucl. Acids Res.* 13: 6223-6236). In the
5 experiment shown in Fig. 2C (upper panel), antisense
and sense RNA preparations (1, 10 and 100 ng) were
applied separately to nitrocellulose sheets. Because
the hybridization signal using the ^{32}P -labelled probe
was sensitive to as little as 1 ng of RNA (Fig. 2C,
10 upper panel), any contaminating opposite sense RNAs
representing 1% or greater of the 100 ng sample
applied would have been easily detected. The data
indicated that no opposite sense RNAs could be
15 observed in the reaction products of the R-RNA cDNA
transcribed from either orientation, even after
prolonged exposure of the autoradiogram. This is
further supported by the finding that one of the RNA
probes (sense orientation) showed no hybridization to
poly(A)+ RNA which indicated a complete lack of
20 complementary sequences, while the other probe
(antisense orientation) hybridized efficiently.
Furthermore, since phosphorylation of PKR was observed
after addition of as little as 12.5 ng of R-RNA
transcript (Fig. 1, lane 8) any contaminating RNAs
25 representing 1% or less in amount would be
insufficient to account for this level of
phosphorylation.

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EXAMPLE 3

1 R-RNA cDNA CLONE ANALYSIS AND SYNTHESIS
 AND PURIFICATION OF THE R-RNA TRANSCRIPT

 The R-RNA cDNA (847 bp) was inserted into
the vector pGEM3Zf(±) at the Eco RI site adjacent to
the T7 promoter and subcloned. The orientation of the
5 cDNA with respect to the T7 polymerase promoter was
determined by restriction mapping with Hind III. As
indicated in Figure 2A, a unique Hind III site is
located within the R-RNA cDNA and an additional Hind
III site is located in the vector 3' to the insertion
10 site. The DNA was separated by 1% agarose gel
electrophoresis and the fragments were visualized
after treatment with ethidium bromide (Figure 2B).

 After the R-RNA cDNA was subcloned into the
pGEM-3Zf(-) and pGEM-3Zf(+) vectors as described
15 above, the cDNA was sequenced in opposite directions
by the dideoxy-mediated chain termination method
(Sanger et al., 1977, *Proc. Natl. Acad. Sci. U.S.A.*
74:5463-5467) using Taq DNA polymerase (Promega).
Reactions contained [α -³⁵S]dATP (3000 Ci/mole,
20 Amersham) and were carried out in the presence of the
universal forward and reverse primers (18-mers)
(Promega). Bidirectional nucleotide sequencing of DNA
amplified by PCR was as described above except that
the amplified DNA was first cloned into the pGEM
25 vector (Promega) containing both the T7 and SP6
promoters. A sequence search of the GenBank data base
using the Wisconsin Genetic Computer Group software
package was conducted. The complete nucleotide
sequence (847 bp) of the partial R-RNA cDNA was
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determined (Fig. 3A) (SEQ ID NO:1). A search of the data base revealed that this sequence had no significant homology to the human or mouse PKR cDNA. Analysis of the nucleotide sequence indicates only one possible uninterrupted open reading frame consisting of 282 amino acids (Fig. 3A), indicating the likelihood that the R-RNA is an mRNA. The R-RNA sequence deduced from the sequence of the corresponding cDNA (SEQ ID NO:1) is set forth in SEQ ID NO:2.

To examine further the possibility that the R-RNA is an mRNA, a synthetic peptide corresponding to amino acids 9-CRLVAKEYLDENNPEES-25 (SEQ ID NO:3) (Fig. 3A) was used to prepare a specific antiserum. Peptide synthesis and purification were carried out by automated FMOC solid phase synthesis as previously described (Nekhai, et al., 1996 *Virology* 222:193-200). Rabbit polyclonal antiserum was raised against hemocyanin-conjugated peptide (EDC Conjugation, Pierce) according to standard procedures (HRP Inc.). Preimmune serum was obtained from the same animal prior to antigen administration; western blot analysis was performed as previously described (Warrener, et al., 1991, *Biochem. Biophys. Res. Commun.* 180: 716-723). Western blot analysis using immune- (Fig. 3B) and preimmune- (Fig. 3C) sera indicated that a 62 KDa protein present in extracts from mouse (Fig. 3B, lane 1) and human (Fig. 3B, lane 2) 3T3 cells and from human MG63 osteosarcoma cells (Fig. 3B, lane 3), specifically reacted with antipeptide serum. This protein did not appear to be expressed in rabbit

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reticulocyte lysate (Fig. 3B, lane 5) or in extracts
from *E.coli* (Fig. 3B, lane 4). Since the 62 KDa
1 protein was detected in at least 3 of the 4 eukaryotic
cell extracts but not in a prokaryotic cell, it
suggests that the R-RNA encoding the 62 KDa protein is
a widely expressed eukaryotic mRNA. The function of
5 the protein is unknown. Recently, two sequences
have been identified as human sequences with near
perfect nucleotide identity to the R-RNA sequence of
the present invention. The two sequences are an EST-
10 cDNA sequence from infant brain which is 99% identical
over its entire 362 bp stretch (Khan, et al., 1992,
Nature. Genet. 2: 180-85) and an cDNA which is 100%
identical over a 231 bp overlap with the R-RNA
sequence (Obradovic et al., EMBL Accession No.
15 HO4703). The evolutionary significance of the high
level of nucleotide conservation remains to be
elucidated but may be explained by a requirement for
the R-RNA to encode a functional protein and also to
retain an ability to form a specific structure(s)
necessary for the activation of PKR.

20 The R-RNA transcript was transcribed in
vitro from the recombinant pGEM-3zf(±) plasmid after
linearization with Sma 1 (Promega). Transcription
from the T7 promoter was carried out using the
MegAscript kit (Ambion) according to the
25 manufacturer's instructions. The reaction mix was
separated by electrophoresis on 0.75 mm, 4%
acrylamide/bisacrylamide (19:1) TBE gels, containing
7M urea. The quantity of the RNA synthesized allowed
30 visualization of bands using shadow-casting. The

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1 advantage of this method is that ethidium bromide,
which may interfere with kinase assays can be avoided.
The gel was covered in plastic wrap and placed on top
of an intensifying screen (Kodak). The gel was
exposed to short wave UV light (254 nm). The shadow
5 cast by the RNA was clearly visible. Gel slices
containing the discrete 847 nt R-RNA transcript were
excised, and the RNA eluted overnight in a solution
(300 μ l) containing 2M $\text{CH}_3\text{COONH}_4$, 0.1% SDS and 0.5 mM
EDTA. The RNA was precipitated in ethanol,
10 resuspended in DEPC-treated H_2O and stored under
liquid nitrogen. The gel purified R-RNA transcript
was digested with RNase T1 (Pharmacia) and RNase
V1 (Pharmacia) and utilized for protein kinase
reactions.

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EXAMPLE 4

PCR ANALYSIS

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PCR analysis was performed utilizing DNA from several eukaryotic sources as a template and the specific primer pair designed to amplify a 380 bp region of the R-RNA cDNA fragment. The oligonucleotide primer pairs were selected from the sequence of R-RNA cDNA (see Fig. 3). The upstream primer corresponding to nucleotides 132-151 (5'-GAAAGTGTAGGCTTGTGCA-3') (SEQ ID NO:4) and downstream primer corresponding to nucleotides 492-511 (5'-CAGCATTAGGAGTTGTGCCC-3') (SEQ ID NO:5) were synthesized using a Model 391 DNA synthesizer (Applied Biosystem) according to the manufacturer's instructions. PCR reactions were carried out as previously described, (Kawasaki, E.S. 1990 in M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, (eds) *PCR Protocols: a Guide to Methods and Applications*, pp. 21-27, Academic Press, New York) with some modifications. The reactions were adjusted to contain in a final volume of 50 μ l: 1 X Taq polymerase buffer, 3.5mM MgCl₂, 1.25 unit of Taq polymerase (Promega) and 25 pmol of primer pairs. Amplification was carried out using a Bioscycler Oven (BIOS Corp.) for 35 cycles under the following conditions: denaturation was for 20 sec at 92°C, synthesis was for 20 sec at 72°C, and annealing (20 sec) was carried out sequentially for 2 cycles at 72°C, 70°C, 68°C, 66°C, 64°C, 61°C, 58°C and 21 cycles at 55° C. Aliquots (20 μ l) from each sample were

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1 separated by electrophoresis on 1% agarose gels
containing 0.045 M Tris-borate and 0.001 M EDTA, and
visualized after staining with ethidium bromide.

5 The results shown in Fig. 4A indicate that
genomic DNA from mouse 3T3-F442A and human CEM cells,
and DNA from mouse liver served as a template to
amplify a 380 bp region of the R-RNA cDNA fragment.
Moreover, a 380 bp region was also amplified when
yeast DNA was used as a template for amplification
(Fig. 4A). In addition, each of the amplified DNAs
10 were sequenced and found to be identical to the
original R-RNA cDNA with the exception of a single
reproducible nucleotide change in the human CEM cell
amplified DNA sequence. To rule out the possibility
that contaminating DNA was amplified during the PCR
15 analysis, genomic DNA from liver tissue of sheep, rat
and mouse and from human CEM, 3T3-F442A and yeast
cells was digested with Eco RI and subjected to direct
Southern blot analysis using the R-RNA cDNA probe.
The data clearly indicate that the R-RNA cDNA probe
20 hybridized to one prominent band present in the
digested DNA from each of these sources (Fig. 4B).
The relative size of the hybridizing fragments was
approximately the same for each of the digested DNA
samples examined except for that of the rat which was
diminished. It is likely that the rat sequence is
25 less conserved and this is reflected in an altered
EcoRI site. In contrast, multiple restriction bands
were detected in genomic DNA from human, mouse and
yeast cells following digestion with BamHI or SmaI.
30 These findings indicate that the R-RNA cDNA sequence

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is present in eukaryotes, and that a portion of the
sequence is highly conserved in nature. This is
1 further confirmed by two independent reports
describing short human cDNA sequences with essentially
100% nucleotide identity to the R-RNA sequence over
the region where sequence information was available.
5 (EMBL accession number X55722; Khan, et al., 1992,
Nature Genet. 2: 180-85).

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EXAMPLE 5

1 NORTHERN AND DOT BLOT ANALYSIS

³²P labeled and non-radioactive RNA
transcripts were prepared from each construct using T7
polymerase after linearization with BamHI. RNAs were
5 denatured with formaldehyde (Sambrook, et al., 1989)
before use. For Northern blots, the RNAs were
separated by electrophoresis on a 1%
agarose-formaldehyde gel and transferred to
nitrocellulose paper (Schleicher & Schuell) as
10 described (Sambrook, et al., 1989). For dot blots,
the RNAs were diluted as indicated in Figure 2C and
applied onto nitrocellulose under vacuum. Both blots
were prehybridized for 4 h at 42°C in a solution
15 containing 5X SSPE, 50% (v/v) formamide, 5X Denhardt's
solution and 10% (w/v) dextran sulfate. Hybridization
was for 18 h at 42°C in the same solution containing
denatured salmon sperm DNA (20 µg/ml) and
[α-³²P]dCTP-labeled R-RNA cDNA probe (25 ng/ml)
20 prepared by nick translation (10⁷ - 10⁸ cpm/µg) for
Northern blots (Sambrook et al., 1989) and
[α-³²P]ATP-labeled R-RNA transcripts (10⁷ cpm/µg) for
dot blots (1 x 10⁶ cpm/ml hybridization buffer). An
actin DNA probe of similar size and labeled to the
25 same specific activity as the R-RNA cDNA probe was
used in some experiments in order to compare the
relative amounts of the hybridization signal between
the R-RNA and actin RNA observed in the total
cytoplasmic RNA preparation. Following hybridization,
30 the blots were washed 4 times with a solution

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1 containing 2X SSC and 0.1% SDS for 5 min each at room temperature. This was followed by 2 washes with 0.1X SSC and 0.1% SDS at 60°C. Blots were air dried and subjected to autoradiography.

5 Previous studies have demonstrated that the R-RNA activity initially observed in the total cytoplasmic RNA obtained from confluent cultures of 3T3-F442A cells quantitatively co-purified with the poly(A)⁺ RNA after separation on oligo(dT)-cellulose (Li, et al., 1991, *Eur. J. Biochem.* 195: 41-48). Consistent with this finding are the results of Northern blot analysis which demonstrate that the R-RNA cDNA hybridizes to a distinct RNA of approximately 2 Kb in size present in the poly(A)⁺ RNA (Fig. 5A, Lane 2) and the total cytoplasmic RNA (Fig. 5B, Lane 1), but not in the poly(A)⁻ RNA prepared from 3T3-F442A cells (Fig. 5A, Lane 3; Fig. 2C). Northern blot analysis also indicates that the hybridizing cytoplasmic RNA is of low abundance (relative to actin mRNA) as would be expected for an RNA involved in PKR activation (Fig. 5B, compare Lanes 1 and 2). These data further indicate that the R-RNA cDNA is not full-length and accounts for only about 48% of the R-RNA sequence. Of additional interest was the observation that the R-RNA cDNA probe hybridized to an RNA of approximately 2 Kb in size, present in the poly (A)⁺ RNA fraction prepared from human CEM cells (Fig. 5A, Lane 1). This indicates that the R-RNA is expressed in some human cells and that it has a functional role in cells other than 3T3-F442A mouse fibroblasts.

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EXAMPLE 6

1 EFFECT OF DNA/RNA HYBRIDIZATION ON
 THE PHOSPHORYLATION OF PKR

 In order to address the possibility that the
 screening procedure used to isolate the R-RNA cDNA and
5 identify the R-RNA could have missed some other
 activating RNAs, hybridization experiments were
 performed. Complementary base pairing between the
 R-RNA cDNA and the R-RNA would specifically disrupt
 the secondary structure in the R-RNA which interacts
10 with PKR, but would have no effect on other
 potentially PKR activating RNAs which would remain
 active.

 To carry out DNA/RNA hybridizations, the
 cytoplasmic RNA (2 μ g), the isolated R-RNA (200 ng)
15 and the R-RNA transcript (20 ng) were each mixed
 with a 5-fold excess (wt/wt) of gel purified R-RNA
 cDNA and heated at 95°C for 5 min in the presence of
 100 mM KCl. The mixtures were then allowed to cool
 slowly to room temperature. For controls, parallel
20 hybridization reactions were performed containing: 100
 mM KCl only; the RNAs alone; cDNA alone;
 poly(I)•poly(C) alone; and poly(I)•poly(C) with 5-fold
 excess cDNA. Aliquots (5 μ l) were added to protein
 kinase assays.

25 The data in Fig. 6 demonstrate that under
 the hybridization conditions used, the R-RNA cDNA
 efficiently prevented the phosphorylation of PKR by
 the R-RNA transcript (Fig. 6, Compare Lanes 3 and 4).
 The R-RNA cDNA alone did not cause phosphorylation of
30 the kinase (Fig. 6, Lane 9) or affect the level of its

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phosphorylation by low levels of poly(I)•poly(C) (data not shown). Of particular importance is the finding that the R-RNA cDNA completely prevented the R-RNA activity in the isolated R-RNA (Fig. 6, Compare Lanes 5 and 6), and in the total cytoplasmic RNA preparation (Fig. 6, Compare Lanes 7 and 8). Moreover, the addition of several unrelated DNAs including the pGEM vector DNA to hybridization reactions had no effect on the subsequent level of PKR phosphorylation by these activating RNA preparations (data not shown). This data indicates that a single cellular RNA is largely responsible for the phosphorylation and activation of PKR.

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EXAMPLE 7

PROPERTIES OF THE R-RNA TRANSCRIPT

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A distinct feature of PKR is that low levels of dsRNA are required for its phosphorylation and activation, but high levels of dsRNA prevent phosphorylation and activation (Farrell et al., 1977, *Cell* 11: 187-200; Petryshyn et al., 1975 *J. Biol. Chem.* 250: 409-417). Protein kinase assays were performed in order to determine if high concentrations of poly(I)•poly(C) could also prevent the phosphorylation of PKR by the R-RNA transcript. The assays were performed under the conditions described in Example 2. Addition of the R-RNA transcript (2.5 $\mu\text{g/ml}$) to protein kinase assays resulted in a level of PKR phosphorylation (Fig. 7, Lane 4) similar to that observed with poly(I)•poly(C) (150 ng/ml) (Fig. 7, Lane 2). This phosphorylation was completely prevented by addition of high concentrations (50 $\mu\text{g/ml}$) of poly(I)•poly(C) (Fig. 7, Lanes 3 and 5). These results demonstrate that the R-RNA transcript facilitates PKR phosphorylation in a manner similar to that observed with low levels of viral and synthetic dsRNA (Petryshyn et al., 1984, *J. Biol. Chem.* 259: 1436-14742; Petryshyn et al., 1988, *Proc. Natl. Acad. Sci. USA* 85: 1427-1931; Galabru et al., 1989, *Eur. J. Biochem.* 178:581-589; Edery et al., 1989, *Cell* 56: 303-312; Petryshyn et al., 1975; Bischoff et al., 1989, *Virology* 172:106-115). Moreover, while maximal phosphorylation was observed with concentrations of between 0.5-2.5 $\mu\text{g/ml}$ of added R-RNA transcript,

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concentrations at 10 μ g/ml and above resulted in a concentration dependent prevention of PKR phosphorylation. Thus the transcript also elicits a paradoxical pattern of PKR phosphorylation similar to that observed with those viral and synthetic dsRNAs which activate PKR. To partially characterize the structural features in the R-RNA transcript involved in PKR activation, the R-RNA transcript was subjected to digestion with the ssRNA-specific RNase T1 and the dsRNA-specific RNase V1, and to thermal denaturation. The activity of the transcript was completely abolished by treatment with RNase V1 (Fig. 7, Lane 7) while RNase T1 had no effect on its activity (Fig. 7, Lane 6). Thermal denaturation (100°C for 2 min) of the transcript followed by either slow cooling (Fig. 7, Lane 8) or rapid cooling (Fig. 7, Lane 9) prior to addition to protein kinase assays had no effect on the activity of the transcript. These results indicate that the R-RNA transcript contains one or more dsRNA regions which are necessary for the phosphorylation of PKR. This region(s) appears to be a favorable structure(s) which reanneals following thermal denaturation to retain sufficient dsRNA structure to facilitate PKR phosphorylation. Moreover, these observed effects of heating, high concentrations of poly(I)•poly(C) and RNases T1 and V1 on the activity of the R-RNA transcript are essentially identical to those previously reported using the R-RNA fraction isolated from the total cytoplasmic RNA of 3T3-F442A cells (Li et al., 1991 *Eur. J. Biochem.* 195:41-48). Thus it is likely that the transcript, although

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shorter in length than the isolated R-RNA, retains the
same structural features involved in the activation if
1 PKR.

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EXAMPLE 8

ASSOCIATION OF THE R-RNA WITH PKR

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In order to determine whether the R-RNA was specifically associated with PKR that had been directly isolated from 3T3-F442A cells, PKR was immune precipitated from cell extracts using anti-PKR antibodies. The RNA in the immune complex was isolated and subjected to reverse transcriptase dependent PCR (RT-PCR) analysis. An association would be expected under physiologic conditions where these cells exhibited high levels of kinase activation (Petryshyn, et al., 1984, *J. Biol. Chem.* 259: 14736-14742; Petryshyn, et al., 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85: 1427-1431). It has previously been shown that under these conditions, the kinase was specifically and quantitatively precipitated (Petryshyn, et al., 1988) and that RNA(s) containing the R-RNA activity was specifically associated with PKR in the immune complex (Li, et al., 1991, *Eur. Bio. Chem.* 195 41-48).

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3T3-F442A cell extract (2.4-4.2 mg protein) was incubated with 200 μ l of rabbit anti-3T3-PKR serum (Petryshyn, et al., 1984) on ice for 1 h. The mixture was transferred to a tube containing a 200 μ l pellet of protein A-Sepharose which had been previously washed 3 times with TBS buffer (10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 100 kU/ml aprotinin, and 0.1% Triton X-100) and incubated overnight at 4°C with constant shaking. The mixture was then centrifuged for 1 min in a macrophage

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to collect the precipitate which contained the complex
of PKR and the associated R-RNA. The precipitate was
1 washed 3 times with 500 μ l TBS buffer and resuspended
in 500 μ l TBS buffer. The R-RNA was extracted from the
complex with phenol:chloroform (1:1) followed by an
extraction with chloroform. The R-RNA was pre-
5 cipitated with ethanol and resuspended in 20 μ l H₂O.

RT-PCR analysis was performed as previously
described (Kawasaki, E.S., 1990) with some
modifications RNAs were heated at 95°C for 5 minutes
and chilled on ice. Reverse transcription reactions
10 (20 μ l) contained 1x Taq polymerase buffer (Promega),
3.5 mM MgCl₂, 1mM each of dNTP, 1 unit/ μ l RNasin, 100
pmol oligo (dt) primer and 7.5 units AMV reverse
transcriptase. The mixtures were preincubated for 10
minutes at 23°C, followed by incubation for 60 minutes
15 at 42°C. Reactions were terminated by heating at 95°C
for 10 minutes.

The results shown in Fig. 8 indicate that
RT-PCR reactions containing total cytoplasmic (Fig. 8,
Lane 1) or poly (A)⁺ RNA (Fig. 8, lane 2) gave rise to
20 a single amplified product of approximately 380 bp.
This is the size of the amplified DNA expected from
reactions using this specific primer pair, and was
also observed by direct PCR of the R-RNA cDNA (Fig. 8,
Lane 7). Importantly, the putative R-RNA extracted
25 from immune precipitates containing PKR, gave rise to
a single amplified DNA of the expected size (Fig. 8,
Lane 4). RT-dependent amplification was not observed
in reactions which contained no RNA (Fig. 8, Lane 6)
or when the oligo(dT) primer was omitted (Fig. 8, Lane
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5). As expected, no amplification was observed when
the A' RNA fraction was used as a template in the RT-
1 PCR reaction (Fig. 8, Lane 3). In addition, direct
dot blot analysis of the RNA extracted from immune
precipitates containing PKR also revealed specific
hybridization to the R-RNA cDNA probe (data not
5 shown). While the possibility cannot be excluded that
other non-activating RNAs may have non-specifically
co-precipitated with PKR, the results demonstrate that
the R-RNA is associated with the kinase that had been
directly obtained from 3T3-F442A cells. Furthermore,
10 the association of the R-RNA with PKR in cell extracts
makes it highly unlikely that its activation by the
isolated or transcript R-RNA (Figs. 1 and 6) is due to
artifactual RNA created by isolation or synthesis
procedures. This finding demonstrates a physiologic
15 role for the R-RNA in the activation of PKR.

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EXAMPLE 9

1 ANALYSIS OF THE DOUBLE-STRANDED
CONTENT OF THE R-RNA

Previous studies have shown that activation of PKR by cytoplasmic RNA obtained from 3T3-F442A cells or by the R-RNA transcript obtained from 3T3-F442A cell cDNA is dependent on single-stranded (ss)-RNA containing double-stranded (ds)-structure(Li, et al., 1991, *Eur. Bio. Chem.* 195: 41-48 and Li, et al., 1997, *Mol. Cell. Diff.* (In Press)). To determine the relative extent of ds-structure in the R-RNA transcript involved in activation of PKR, its activity was examined via protein kinase assays over a broad concentration range and compared to activation with poly(I)-poly(C).

Protein kinase assays (20 μ l) using PKR purified from rabbit reticulocytes (0.5 ng) (Pettryshyn, et al., 1983, *Methods Enzymol.* 99: 346-362) were performed under conditions as described (Pettryshyn, et al., 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85: 1427-1431). Other additions are as indicated in Figure 9. Proteins were separately by electrophoresis on 7.5% SDS-polyacrylamide gels (SDS-PAGE) and phosphoprotein profiles were analyzed following autoradiography. Poly(I)-poly(C) was obtained from Pharmacia and [γ -³²P]ATP(4500Ci/mmol) was obtained from Dupont. The extend of PKR phosphorylation was quantitated from autoradiograms by scanning densitometry (Shimadzu, Kyoto, Japan).

30 The R-RNA transcript and poly(I)-poly(C) were added to the protein kinase assays at the final

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concentrations indicated in Figure 9, and the extent of activation was determined by quantitation of the level of PKR phosphorylation. The results show that the profile of activation observed with the R-RNA transcript was similar to that observed with poly(I)-poly(C) (Fig.9). Both RNAs show a transient effect on PKR, i.e., a dose dependent activation at low concentration followed by a dose dependent inhibition of activation at higher concentrations. The transient effect of poly(I)-poly(C) observed is consistent with previous reports (Farrell et al., 1977 *Cell* 11: 187-200; Hunter et al., 1975, *J. Biol. Chem.* 250: 409-417). However, the minimum concentration of the R-RNA transcript (0.64 ug/ml, dashed line, Fig.9), necessary for optimal activation of PKR is 16-fold higher than the minimal concentration of poly(I)-poly(C) required for a similar level of activation, (0.04 ug/ml, dashed line, Fig. 9). Since poly(I)-poly(C) can be assumed to be entirely composed of ds-structure (Hunter, et al., 1975, *J. Bio. Chem.* 250: 409-417), and the R-RNA transcript contain both ss- and ds-structures (Li, et al., 1997, *Mol. Cell. Diff. (In Press)*), the difference in the relative minimum concentrations needed for optimal activation may represent differences in the extent of ds-structure between the two molecules. This implies that the ds-region involved in activation of PKR constitutes a small portion of the secondary structure with the R-RNA. This is further supported by the observation that digestion of the transcript with ss-RNA specific RNase T1 resulted in formation of RNA fragments of

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1 approximately 60 bp or less in length (Fig. 10A, lanes
3-7). These fragments are still capable of activating
PKR (Fig. 10B, lanes 3-7) indicating that they retain
the critical ds-structure(s) even after digestion with
high levels of RNase T1. Digestion with ds-RNA
5 specific RNase VI however, results in formation of
fragments of a larger average size (Fig. 10A, lanes 8-
12) that are unable to facilitate activation of PKR
(Fig. 10B, lanes 8-12).

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EXAMPLE 101 **MAPPING OF SECONDARY-STRUCTURE INVOLVED
 IN ACTIVATING PKR**

 To delineate the location of the region of
secondary structure and nucleotide sequence of R-RNA
involved in activation of PKR, RNA:DNA hybridization
5 reactions were carried out utilizing the R-RNA
transcript and specific restriction fragments from its
cDNA. The mappings of the secondary-structure within
the R-RNA important for activation of PKR can be
established by determining which of the known
10 restriction fragments interfere with the capacity of
the R-RNA to activate PKR. This is because such
RNA:DNA duplexes are stable once they form, and they
disrupt the natural secondary-structure of the R-RNA.
Digestion with Alu I was selected because such
15 digestion gave rise to five asymmetric fragments which
could be conveniently separated and four of the
fragments were easily purified.

 Figure 11A is a schematic representation
indicating the known restriction map of the 847 bp R-
20 RNA cDNA and the orientation for the five predicted
Alu I fragments. Following digestion with Alu I, the
predicted 284, 226, 178 and 133 bp fragments were
obtained and highly purified by gel electrophoresis.

 To prepare highly purified R-RNA cDNA
25 insert, isolated pGEM-3zf(±) recombinant plasmid was
digested with EcoR1 (Li et al., 1997, Mol. Cell. Diff.
(In press.)) and R-RNA cDNA (1mg) insert was separated
by electrophoresis on 1.2% low melting temperature
30 agarose (NuSieve, FMC Bioproducts). The 847 bp R-RNA

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1 cDNA was purified from gel slices using the QIAEXII,
extraction kit (Qiagen). The purified cDNA was
digested at 37° C with Alu I (NEB). The reaction
productions were separated by electrophoresis on 1.5%
5 agarose gels and the expected 284, 226, 173 and 133 bp
fragments were individually purified as described
above. The expected 26 bp fragment was not recovered.
The purified cDNA fragments were resuspended in DEPC-
treated H₂O and stored at -20° C at a concentration of
20-25 ug/ml.

10 The purity of the individual fragments is
shown in figure 11B. Each cDNA fragment was
hybridized to the R-RNA transcript individually or in
combination. For RNA/DNA hybridization assays (10µl)
reactions contained 100 mM KCl, 0.1 mM EDTA (pH 7.0),
15 R-RNA transcript (5 ng) or poly(I)-poly(C) (3 ng), and
were supplemented with a 10-fold molar excess of Alu I
DNA fragments or 50-100 ng of complementary oligo as
indicated in Figures 11C, 12B, and 12C. RNase/DNase-
free non-specific *E.coli* DNA was added to reactions as
20 a carrier to minimize loss of RNA transcript during
hybridization. Hybridization reactions were for 5
minutes at 95° in tightly capped tubes. The mixtures
were allowed to slow cool (30-60 min) to room
temperature. Reactions were placed on ice and used
25 directly for protein kinase assays. Controls included
parallel hybridization reactions containing:
hybridization buffer alone; Alu I fragments alone;
oligos alone; poly(I)-poly(C) alone; poly(I)-poly(C)
with excess Alu I fragments; and poly(I)-poly(C) with

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oligos (100 ng) as indicated in Figures 11C, 12B, and 12C. All control reactions contained carrier DNA.

1 The effect of hybridization of activation of
PKR was analyzed utilizing protein kinase assays as
described in Example 9. It was determined that the
226 bp fragment alone could prevent the R-RNA
5 transcript from activating PKR (Fig. 11C, compare
lanes 3 and 5). The 284, 178 and 133 bp fragments had
little or no effect on the R-RNA activity (Fig. 11C,
lanes 4, 6 and 7). Moreover, in mixing experiments
10 containing all possible combinations of the Alu I
fragments, PKR activation was prevented in only those
assays which contained the 226 bp fragment (Fig. 11C,
lanes 8-14). No phosphorylation of PKR was observed
in the absence of ds-RNA (Fig. 11C, lane 1). Controls
15 for hybridization/kinase assays included reactions
which contained only poly(I)-poly(C) (Fig. 11C, lane 2),
and the R-RNA transcript (Fig. 11C, lane 3). The
phosphoprotein observed on autoradiograms of lower
molecular weight than PKR represent trace amounts of
contaminating proteins that vary in PKR preparations.
20 Their phosphorylation is not dependent on RNA and is
not affected by the Alu I fragments. The effect of
the 226 bp fragments was specific because activation
of PKR by poly(I)-poly(C) was not affected by addition
of the 226 pb fragments to hybridization reactions and
25 the 226 did not effect the phosphorylation of PKR by
the R-RNA without being subjected to hybridization
conditions. Moreover, no measurable ribonuclease
activity was detected in the 226 pb Alu I fragment to
account for loss of RNA (Fig. 11D, lane 3) during
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hybridization. As an additional control, the 284 bp
Alu I fragment was also examined and found to contain
1 no ribonuclease activity (Fig. 11D, lane 2). The 26 bp
fragment could not be recovered from gels and directly
tested in hybridization reactions. However, in
reactions where limited digestion of the 847 bp R-RNA
5 cDNA with Alu I was carried out, a 252 bp fragment
identified by additional restriction mapping with Xho
I (IBI) as consisting of the 226 + 26 bp (Fig. 3A)
fragment was obtained and purified. The 252 bp
10 fragment was equally effective in preventing PKR
activation as the 226 bp fragment following
hybridization of the R-RNA (data not shown). These
findings indicate that the secondary structures(s)
important for the activation of PKR are localized to
the 226/252 nt region within the R-RNA transcript
15 consisting of nucleotides 178-430.

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EXAMPLE 11

1 **EFFECT OF ANTISENSE OLIGODEOXYNUCLEOTIDES
ON THE ACTIVITY OF THE R-RNA**

 In order to further define the region(s)
within the 252 nt fragment of the R-RNA involved in
5 activation of PKR, a novel antisense approach was
developed. Eleven short phosphorothioate
oligodeoxynucleotides (20-22 nt in length), the sum of
which are complementary to the 252 nt stretch of the
R-RNA were synthesized. The oligonucleotides were
10 selected for optimal hybridization, and minimal self-
folding and homo-dimer annealing. The sequence and
size of the oligos used were:

OL1A	5'-CTCATCTAGCTTATCCATT-3'	20 mer	(SEQ ID NO:6)
OL2A	5'-ATCCGGTTCATACGCCTCATC-3'	21 mer	(SEQ ID NO:7)
OL-1	5'-CCTGCAATGATTCCAATTCC-3'	20 mer	(SEQ ID NO:8)
15 OL3A	5'-ATTTAATTCAATAGATGCATAT-3'	22 mer	(SEQ ID NO:9)
OL-2	5'-TATTGGACGAATGCATTTTG-3'	20 mer	(SEQ ID NO:10)
OL4A	5'-TCTTCCTTTGATAGCGACCT-3'	20 mer	(SEQ ID NO:11)
OL-3	5'-GAGTAATCAGGATCTTCCTT-3'	20 mer	(SEQ ID NO:12)
OL5A	5'-CCTTATGACTTGACCCTCTATA-3'	22 mer	(SEQ ID NO:13)
20 OL-4	5'-AGGAGTTGTGCCCAGTCCCA-3'	20 mer	(SEQ ID NO:14)
OL6A	5'-TTCATTCTTTTAGCTGACAGC-3'	21 mer	(SEQ ID NO:15)
OL7A	5'-CTCAATATCTAGCTTAAATG-3'	20 mer	(SEQ ID NO:16)
OLRS	5'-GTTTTACGTAAGCAGGTTAT-3'	20 mer	(SEQ ID NO:17)
OLRD	5'-AATGTGTAGTTTCGTACTGA-3'	20 mer	(SEQ ID NO:18)

25 Figure 12A depicts the location and spatial
arrangement of the oligos in respect to the R-RNA.
The effect of hybridization of each of these oligos to
the R-RNA on the activation of PKR was examined using
30 protein kinase assays. The results indicate that

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oligos OL1A (SEQ ID NO:6) (Fig. 12B, lane 4), OL2A (SEQ
ID NO:7) (Fig. 12, lane 5), OL-2 (SEQ ID NO:10) (Fig.
1 12B, lane 8) and OL-4 (SEQ ID NO:14) (Fig. 12B, lane
12) were effective in blocking the activation of PKR.
The remaining seven oligos had a modest and variable
inhibitory effect at identical concentrations, which
5 may be due to nonspecific inhibition because a similar
level of reduction was observed with non-complementary
oligos (OLRS [SEQ ID NO:17] and OLRD [SEQ ID NO:18]).
The largest gap between oligos was 23 nt (between OL-2
[SEQ ID NO:10] and OL4A [SEQ ID NO:11]), which alone is
10 insufficient in length for activation of PKR (Manche,
et al. 1992, *Mol. cell. Biol.* 12: 5238-5248). The
possibility however, that nucleotides in the gap
sequence contributed to other sequences in the 226/252
region for activation of PKR cannot be absolutely
15 excluded. PKR was not phosphorylated in the absence
of RNA (Fig. 12B, lane 1). Controls included one
assay which contained only poly(I)-poly(C) (Fig. 12B,
lane 2) and one assay which contained only R-RNA
transcript (Fig. 12B, lane 3). The effects of these
20 blocking oligos appears specific because they had no
effect on activation by poly(I)-poly(C) (Fig. 12C,
compare lane 2 and lanes 3-6). Moreover, no
ribonuclease activity could be detected in blocking
oligo preparations, or loss of R-RNA due to addition
25 of these oligos (Fig. 13 compare lane 1 and lanes 2-
5). These findings suggest nucleotide sequences 178-
202, 263-283 and 374-393 within the R-RNA transcript
are involved in the activation of PKR which can be

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prevented by hybridization to corresponding
complementary oligos.

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EXAMPLE 12

EFFECT OF OL-2 ON MURINE EMBRYONIC 3T3-F442A FIBROBLASTS

The effect of OL-2 (SEQ ID NO:10) was tested on a variety of cell types and heterogenous cell populations for effects on proliferation, differentiation, and viability. Pre-confluent (log-phase of growth), 3T3-F442A cells cultured under normal conditions and media (10% FBS/DMEM) were treated separately with OL-2 (SEQ ID NO:10) and 2 distinct control oligos. The control oligonucleotides consisted of OLRD (SEQ ID NO:18) and an oligonucleotide having the sequence:

5' CCTCGGTCCCCCTCGTCCC 3' (SEQ ID NO:19).

OL-2 (SEQ ID NO:10) was added directly to a cell culture of 3T3-F442A cells to achieve a final concentration of 1.0-10µM. After three days and again after five days in culture , cells were examined by microscopy and cell numbers were determined by counting using a hemocytometer. As depicted in Fig.14, it was observed that OL-2 (SEQ ID NO:10) stimulated a 2-3 fold increase in cell number compared to controls resulting in a corresponding increase in saturation density. This increase in saturation density was observed at least between 1.0 -10 µM concentrations of OL-2 (SEQ ID NO:10) and was concentration dependent. No toxicity was observed in cells treated with up to 10 µM final concentration.

Cell uptake analysis, performed by the method described in Crooke et al. 1995, *J. Pharm. Exp. Ther.* 275:462-473, indicated that radio-labeled OL-2

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(SEQ ID NO:10) was stable and intact intracellularly
for up to 72 hours. These results indicate that OL-2
1 (SEQ ID NO:10) specifically stimulates an increase in
cell density as a result of continued cell
proliferation in embryonic cells.

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EXAMPLE 13EFFECT OF OL-2 ON HUMAN
PERIPHERAL BLOOD MONONUCLEAR CELLS

1 Fresh drawn human peripheral blood was
layered onto Ficoll-Hypaque gradients and centrifuged.
Unfractionated mononuclear cells were collected and
5 treated with antisense OL-2 (SEQ ID NO:10) and control
oligos at final concentrations of 0.5-5 uM. Cells
were exposed to the oligos for three days in
suspension medium containing Iscoves Modified Eagle's
Medium (IMDM) and low serum level, under normal
10 culture conditions. The oligo-treated and control
cells were cultured on methylcellulose plates
containing erythropoietin, or GM-CSF. Burst-forming
units of erythroid lineage (BFU-E) or colony-forming
units of granulocyte/macrophage lineage (CFU-GM) were
15 determined by microscopic examination following ten
days and 18 days of culture in methylcellulose.

Figures 15 through 17 illustrate the effect
of OL-2 (SEQ ID NO:10) on erythroid progenitors
derived from peripheral blood mononuclear cells
20 (PBMC). Controls include medium alone (no
oligonucleotides) and an unrelated oligonucleotide
having the sequence set forth in SEQ ID NO:19.

Figure 15 is a graph which depicts results
of a representative experiment summarizing the
25 response of four PBMC samples drawn from three
individuals. PMBC's were incubated with the
oligonucleotides for three days, then analyzed for
burst forming units - erythroid (BFU-E's), the most
primitive erythroid progenitors than can be currently
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analyzed. Results are expressed as the number of
bursts counted per plate (average of triplicate
plates). The data indicate that one of the
oligonucleotides, OL-2 (SEQ ID NO:10), promotes an
approximate 2X increase in burst numbers at a
concentration of 1 uM. In addition, cells treated
with OL-2 (SEQ ID NO:10) formed larger colonies,
indicating the presence of greater cell numbers per
colony.

Figure 16 is a graph which depicts results
from an identical assay and demonstrates a dose-
dependent increase in burst number. Effects are seen
with doses as low as 0.5 uM (1-5x increase). At a
dose of 5uM (the highest tested), OL-2 (SEQ ID NO:10)
exhibited approximately a 4x increase in burst number.
No effect was observed in cells treated with the other
oligonucleotides (OL-1, OL-2, or OL-4) or a control
oligonucleotides having the sequence set forth in SEQ
ID NO:19, even at a dose of 5 uM.

As depicted in Figure 17, significant
increases in BFU-E's (1.5x) were observed between 24
to 48 hours after addition of OL-2 at 1uM. Increases
(2.5x) were also observed up to 72 hours, the longest
time point examined. These results indicate that a
time period of between 24-48 hours is sufficient to
accumulate an effective intracellular level of OL-2
(SEQ ID NO:10).

Thus, OL-2 (SEQ ID NO:10), significantly and
specifically promotes an increase in BFU-E numbers.
These results indicate that OL-2 (SEQ ID NO:10)

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selectively increases the population of early
progenitor cells (hematopoietic stem cells).

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EXAMPLE 141 **EFFECT OF OL-2 ON PRIMARY HUMAN MARROW CELLS**

Frozen SBA negative (T and B cell depleted) human bone marrow cells (1×10^7 cell/vial) were thawed and washed with 2% FBS in IMDM. Cells were

5 resuspended at 1×10^6 cells per ml in long term culture-initiating cell medium (LTC-IC) consisting of 12.5% fetal bovine serum (FBS), 12.5% horse serum, 10^{-6} M hydrocortisone in IMDM medium. Cells were plated in 48-well plates at 0.5ml/well to give a cell

10 concentration of 5×10^5 cells/well. OL-2 and control oligos were added to achieve a final concentration of 5 μ M/well. Triplicate samples were used for each condition. Oligos were replaced 2-times weekly for three weeks.

15 After three weeks, cultures were harvested by treatment with collagenase and resuspended in LT-IC medium and re-plated on methylcellulose plates at 4×10^4 cells/well. After 3-4 weeks of culture, BFU-Es, CFU-GMs, CFU-GEMM and total CFUs were determined

20 following microscopic examination. Table 1 summarizes the data for one LTC-IC assay. It is concluded that OL-2 (SEQ ID NO:10) and OL-1 (SEQ ID NO:8) alter the cellularity of primary human bone marrow cells. Burst-forming units-erythroid (BRU-E); colony-forming

25 units-granulocyte/ macrophage (CFU-GM); colony-forming units-granulocyte/ erythroid/myeloid/megakaryocyte (CFU-GEMM) are consistently increased 2.5-7 times with a total cellular increase of approximately 3 fold. No toxicity was observed. The increase in colonies of

30 all major lineages in response to OL-2 (SEQ ID NO:10)

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and OL-1 (SEQ ID NO:8) indicates an expansion of the pluripotent progenitor cell(s).

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TABLE 1

EFFECT OF ANTISENSE OLIGONUCLEOTIDES
ON THE CELLULARITY OF HUMAN BONE MARROW

	BFU-E	CFU-GM	CFU-GEMM	TOTAL CFU
control	1	18	2	21
OL-1	7	55	5	67
OL-2	7	55	7	69

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EXAMPLE 151 **EFFECT OF OL-2 ON THE CELLULARITY OF MARROW FROM A
PATIENT WITH SEVERE CONGENITAL NEUTROPENIA**

5 Fresh marrow was obtained with consent from
a patient with Severe Congenital Neutropenia (SCN).
Cells were separated by Ficoll-Hypaque centrifugation.
5 The buffy coat was collected and resuspended in McCoys
media. Cell suspensions (5×10^5 cells/ml) were treated
with OL-2 (SEQ ID NO:10) ($5 \mu\text{M}$), a control oligo, or
received no treatment, for 70-245 min. Following this
10 procedure, the cells were plated in methylcellulose
LTC-IC media containing granulocyte/macrophage-colony
stimulating factor (GM-CSF) at a concentration of 20-
100 ng/ml. Neutrophils were determined after 8-14
days by microscopic inspection and by the specific
15 neutrophil staining for lactoferrin and lactase. It
was determined that untreated cells contained no
detectable neutrophils. In contrast, marrow treated
with OL-2 contained clearly recognizable neutrophils.
These results indicate that OL-2 (SEQ ID NO:10)
20 promotes neutrophil expansion and development from a
neutrophil depleted SCN marrow. This finding also
indicates a clinical benefit for OL-2 (SEQ ID NO:10)
in treatment of SCN.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Research Corporation Technologies, Inc.
- (ii) TITLE OF INVENTION: CELL GROWTH CONTROLLING
OLIGONUCLEOTIDES
- (iii) NUMBER OF SEQUENCES: 19
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Scully, Scott, Murphy & Presser
 - (B) STREET: 400 Garden City Plaza
 - (C) CITY: Garden City
 - (D) STATE: New York
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 11530
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: DiGiglio, Frank S.
 - (B) REGISTRATION NUMBER: 31,346
 - (C) REFERENCE/DOCKET NUMBER: 10303
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 516-742-4343
 - (B) TELEFAX: 516-742-4366
 - (C) TELEX: 230 901 SANS UR

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 847 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

- 85 -

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGCACAAGGT GACATTTTAA GAAAGTGTAG GCTTGTCGCA AAGGAGTATT TAGATGAAAA	60
TAACCCAGAA GAATCAATTG GTGATCTTCA ATTCAATTTG AATATCTCAG AAATAGAAAA	120
TAATATAGTA TCACCTCTTG AACGCTCAGA CAGGAAAGTT GTCATATTAA TGGATAAGCT	180
AGATGAGGCG TATGAACCGG ATAATATAGG AATTGGAATC ATTGCAGGTC TAGCATATGC	240
ATCTATTGAA TTAAATCAAA AAGCAAAATG CATTCGTCCA ATAATTTTTT TAAGGGATAA	300
TATATTTAGG TCGCTATCAA AGGAAGATCC TGATTACTCG AGAAATATAG AGGGTCAAGT	360
CATAAGGTTG CATTGGGACT GGGCACAACT CCTAATGCTG TCAGCTAAAA GAATGAAAGT	420
AGCATTTAAG CTAGATATTG AGAAAGATCA ACGAGTTTGG GATAGATGCA CAGCGGATGA	480
TCTTAAAGGG AGGATTGGTT TTAAGCGATG TTTGCAATTT ACCCTCTACC GGCCAGGGA	540
TTTACTATCA TTGTTGAATG AAGCTTTTTT TTCCGCATTC AGAGAGAATA GAGAACTAT	600
CATAAACACT GACCTAGAAT ATGCAGCCAA GTCAATTTCC ATGGCCAGAC TTGAAGATCT	660
CTGGAAAGAG TATCAGAAGA TCTTTCCTTC AATACAGGTT ATAAC TAGTG CATTTCGCAG	720
CATTGAACCT GAATTAACAG TTTATACGTG CTTAAAAAAA ATAGAAGCAT CTTTCGAATT	780
AATCGAAGAA AATGGAGATC CTAAAATAAC GTCTGAAATA CAGTTGTTAA AGGCAAGTGG	840
AATTCCG	847

- 86 -

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 847 nucleotides
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

UGCACAAGGU GACAUUUUAA GAAAGUGUAG GCUUGUCGCA AAGGAGUAAU UAGAUGAAAA	60
UAACCCAGAA GAAUCAAUUG GUGAUCUUCA AUUCAAUUUG AAUAUCUCAG AAUAGAAAA	120
UAAUAUAGUA UCACUUCUUG AACGCUCAGA CAGGAAAGUU GUCAGAUUAA UGGAUAAGCU	180
AGAUGAGGCG UAUGAACCGG AUAAUAUAGG AAUUGGAAUC AUUGCAGGUC UAGCAUAUGC	240
AUCUAUUGAA UAAAAUCAA AAGCAAAAUG CAUUCGUCCA AUAAUUUUUU UAAGGGAUAA	300
UAUAUUUAGG UCGCUAUCAA AGGAAGAUCC UGAUUACUCG AGAAUAUAG AGGGUCAAGU	360
CAUAAGGUUG CAUUGGGACU GGGCACAACU CCUAAUGCUG UCAGCUAAAA GAAUGAAAGU	420
AGCAUUUAAG CUAGAUAUUG AGAAAGAUCA ACGAGUUUGG GAUAGAUGCA CAGCGGAUGA	480
UCUAAAAGGG AGGAAUGGUU UUAAGCGAUG UUUGCAAUUU ACCCUUUACC GGCCAGGGA	540
UUUACUAUCA UUGUUGAUG AAGCUUUUUU UUCCGCAUUC AGAGAGAAUA GAGAAACUUA	600
CAUAAACACU GACCUAGAAU AUGCAGCCAA GUCAAUUUCC AUGGCCAGAC UUGAAGAUCU	660
CUGGAAAGAG UAUCAGAAGA UCUUCCUUC AAUACAGGUU AUAACUAGUG CAUUUCGUAG	720
CAUUGAACCU GAUUUAACAG UUUUAUCGUG CUUAAAAAAA AUAGAAGCAU CUUUCGAAUU	780
AAUCGAAGAA AAUGGAGAUC CUAAAUAAC GUCUGAAUA CAGUUGUUA AGGCAAGUGG	840
AAUCCG	847

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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Cys Arg Leu Val Ala Lys Glu Tyr Leu Asp Glu Asn Asn Pro Glu Glu
1 5 10 15
Ser

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAAAGTGTAG GCTTGTGCGA

20

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAGCATTAGG AGTTGTGCCC

20

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(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCTCATCTAG CTTATCCATT

20

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCCGGTTCA TACGCCTCAT C

21

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCTGCAATGA TTCCAATTCC

20

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(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATTTAATTCA ATAGATGCAT AT

22

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TATTGGACGA ATGCATTTTG

20

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCTTCCTTTG ATAGCGACCT

20

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(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAGTAATCAG GATCTTTCTT

20

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCTTATGACT TGACCCTCTA TA

22

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGGAGTTGTG CCGACTCCCA

20

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(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTCATTCTTT TAGCTGACAG C

21

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTCAATATCT AGCTTAAATG

20

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTTTTACGTA AGCAGGTAT

20

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(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AATGTGTAGT TGCCTACTGA

20

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCTCGGTCCC CCCTCGTCCC

20

93

WHAT IS CLAIMED IS:

1

1. An isolated nucleic acid molecule consisting of a nucleotide sequence or complementary to a nucleotide sequence as set forth in SEQ ID NO:1 or having at least 50% similarity or complementarity thereto.

5

2. An isolated nucleic acid molecule consisting of a nucleotide sequence or complementary to a nucleotide sequence as set forth in SEQ ID NO:2 or having at least 50% similarity or complementarity thereto.

10

3. An isolated nucleic acid molecule consisting of or complementary to nucleotides 178-430 of SEQ ID NOS:1 or 2 or having at least 50% similarity or complementarity thereto.

15

4. An isolated nucleic acid molecule which hybridizes to nucleotides 178-430 of SEQ ID NO:2 under medium to high stringency conditions.

5. An isolated nucleic acid molecule consisting of or complementary to nucleotides 263-283 of SEQ ID NOS:1 or 2 or having at least 50% similarity or complementarity thereto.

20

6. An isolated nucleic acid molecule which hybridizes to nucleotides 263-283 of SEQ ID NO:2 under medium to high stringency conditions.

25

7. An isolated nucleic acid molecule consisting of or complementary to nucleotides 374-393 of SEQ ID NOS:1 or 2 or having at least 50% similarity or complementarity thereto.

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1 8. An isolated nucleic acid molecule which hybridizes to nucleotides 374-393 of SEQ ID NO:2 under medium to high stringency conditions.

9. An oligonucleotide comprising the sequence set forth in SEQ ID No. 6.

5 10. An oligonucleotide comprising at least eight contiguous nucleotides derived from the sequence set forth in SEQ ID NO:6.

11. An oligonucleotide comprising the sequence set forth in SEQ ID No. 7.

10 12. An oligonucleotide comprising at least eight contiguous nucleotides derived from the sequence set forth in SEQ ID NO:7.

13. An oligonucleotide comprising the sequence set forth in SEQ ID No. 10.

15 14. An oligonucleotide comprising at least eight contiguous nucleotides derived from the sequence set forth in SEQ ID NO:10.

15. An oligonucleotide comprising the sequence set forth in SEQ ID No.14.

20 16. An oligonucleotide comprising at least eight contiguous nucleotides derived from the sequence set forth in SEQ ID NO:14.

25 17. An oligonucleotide comprising at least eight nucleotides which are complementary to at least eight contiguous nucleotides of nuceotides 178-430 as set forth in SEQ ID NO:1.

30 18. A method of stimulating cell proliferation in a cell culture which comprises contacting said cell culture with an effective amount of an oligonucleotide comprising the sequence set

95

forth in SEQ ID NO:10 or at least eight contiguous nucleotides of SEQ ID NO:10.

1

19. A method of inhibiting the activation of PKR in a cell culture which comprises contacting said cell culture with an effective amount of at least one of an isolated nucleic acid molecule complementary to SEQ ID NO:1, an isolated nucleic acid molecule complementary to nucleotides 178-430 of SEQ ID NOS:1 or 2, an isolated nucleic acid molecule complementary to nucleotides 263-283 of SEQ ID NOS:1 or 2, an isolated nucleic acid molecule complementary to nucleotides 374-393 of SEQ ID NOS:1 or 2; an oligonucleotide having the sequence set forth in SEQ ID NO:6 or at least eight contiguous nucleotides of SEQ ID NO:6, an oligonucleotide having the sequence set forth in SEQ ID NO:7 or at least eight contiguous nucleotides of SEQ ID NO:7, an oligonucleotide having the sequence set forth in SEQ ID NO:10 or at least eight contiguous nucleotides of SEQ ID NO:10, or an oligonucleotide having the sequence set forth in SEQ ID NO:14 or at least eight contiguous nucleotides of SEQ ID NO:14.

5

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20. A pharmaceutical composition capable of inhibiting activation of PKR in a cell culture which comprises at least one of the isolated nucleic acid molecules or oligonucleotides of Claim 19 admixed with a pharmaceutically acceptable carrier.

25

21. A pharmaceutical composition capable of stimulating cell proliferation in an a cell culture which comprises an oligonucleotide having the sequence set forth in SEQ ID NO:10 or at least eight contiguous

30

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96

1 nucleotides of SEQ ID NO:10 admixed with a
pharmaceutically acceptable carrier.

22. A kit comprising at least one of the
oligonucleotides of claims 9-17 and a pharmaceutically
acceptable carrier.

5 23. The kit of claim 22 wherein the
pharmaceutically acceptable carrier is packaged
separately from the oligonucleotides.

24. The kit of claim 22 wherein the
pharmaceutically acceptable carrier is adm the R-RNA
cDNA ixed with the oligonucleotides.

10 25. A kit comprising at least one of the
isolated nucleic acids of Claims 1-8 and a
pharmaceutically acceptable carrier.

26. The kit of claim 25 wherein the
pharmaceutically acceptable carrier is packaged
separately from the isolated nucleic acid molecules.

27. The kit of claim 25 wherein the
pharmaceutically acceptable carrier is admixed with
the isolated nucleic acid molecules.

20 28. A method of inhibiting cell
proliferation in bone marrow cells obtained from a
patient suffering from a hematological cancer which
comprises isolating a bone marrow sample from a
patient suffering from a hematological cancer,
contacting the cells in said sample with an effective
25 amount of at least one of an R-RNA having the sequence
set forth in SEQ ID NO:2, a portion of an R-RNA having
nucleotides 178-430 of SEQ ID NO:2, a portion of an R-
RNA having nucleotides 263-283 of SEQ ID NO:2, or a
portion of an R-RNA having nucleotides 374-393 of SEQ
30

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1 ID NO:2, and after a sufficient time, transplanting
said cells back into the donor patient.

5 29. A method for promoting expansion of
pluripotent progenitor cells which comprises obtaining
bone marrow cells from a patient and contacting said
cells with an effective amount of an oligonucleotide
selected from the group consisting of an
oligonucleotide having the nucleotide sequence set
forth in SEQ ID NO:10, an oligonucleotide having at
least eight contiguous nucleotides of SEQ ID NO:10, an
oligonucleotide having the nucleotide sequence set
10 forth in SEQ ID NO:8, and an oligonucleotide having at
least eight contiguous nucleotides of SEQ ID NO:8.

15 30. A method for promoting expansion of
hematopoietic stem cells which comprises obtaining
peripheral blood from a patient; isolating mononuclear
cells and contacting said mononuclear cells with an
effective amount of an oligonucleotide having the
nucleotide sequence set forth in SEQ ID NO:10 or at
least eight contiguous nucleotides of SEQ ID NO:10.

20 31. A method for promoting neutrophil
exansion and development from a neutrophil depleted
marrow cell culture which comprises obtaining marrow
cells from a patient suffering from severe congenital
neutropenia (SCN), contacting said cells with an
effective amount of an oligonucleotide having the
25 sequence set forth in SEQ ID NO:10 or at least eight
contiguous nucleotides of SEQ ID NO:10, and after a
sufficient time, transplanting the marrow cells back
into the patient.

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32. A method for expanding hematopoietic
cells in umbilical cord blood which comprises
1 contacting a sample of the cord blood with at least
one oligonucleotide selected from the group consisting
of an oligonucleotide having the sequence set forth in
SEQ ID NO:8, an oligonucleotide having at least eight
5 contiguous nucleotides as set forth in SEQ ID NO:8, an
oligonucleotide having the sequence set forth in SEQ
ID NO:10 or an oligonucleotide having at least eight
contiguous nucleotides as set forth in SEQ ID NO:10.

33. A composition comprising a cell culture
10 medium and at least one of a nucleic acid molecule or
oligonucleotide admixed with said cell culture medium
selected from the group of an isolated nucleic acid
molecule complementary to SEQ ID NO:1, an isolated
nucleic acid molecule complementary to nucleotides
15 178-430 of SEQ ID NOS:1 or 2, an isolated nucleic acid
molecule complementary to nucleotides 263-283 of SEQ
ID NOS:1 or 2, an isolated nucleic acid molecule
complementary to nucleotides 374-393 of SEQ ID NOS:1
or 2; an oligonucleotide having the sequence set forth
20 in SEQ ID NO:6 or at least eight contiguous
nucleotides of SEQ ID NO:6, an oligonucleotide having
the sequence set forth in SEQ ID NO:7 or at least
eight contiguous nucleotides of SEQ ID NO:7, an
oligonucleotide having the sequence set forth in SEQ
25 ID NO:10 or at least eight contiguous nucleotides of
SEQ ID NO:10, or an oligonucleotide having the
sequence set forth in SEQ ID NO:14 or at least eight
contiguous nucleotides of SEQ ID NO:14.

30

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FIG.1A

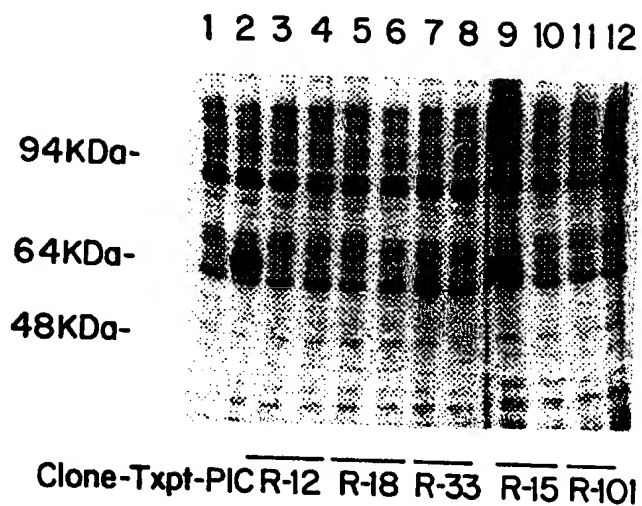
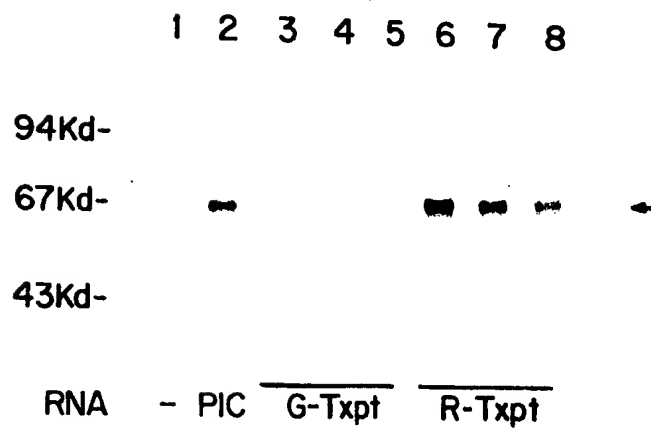
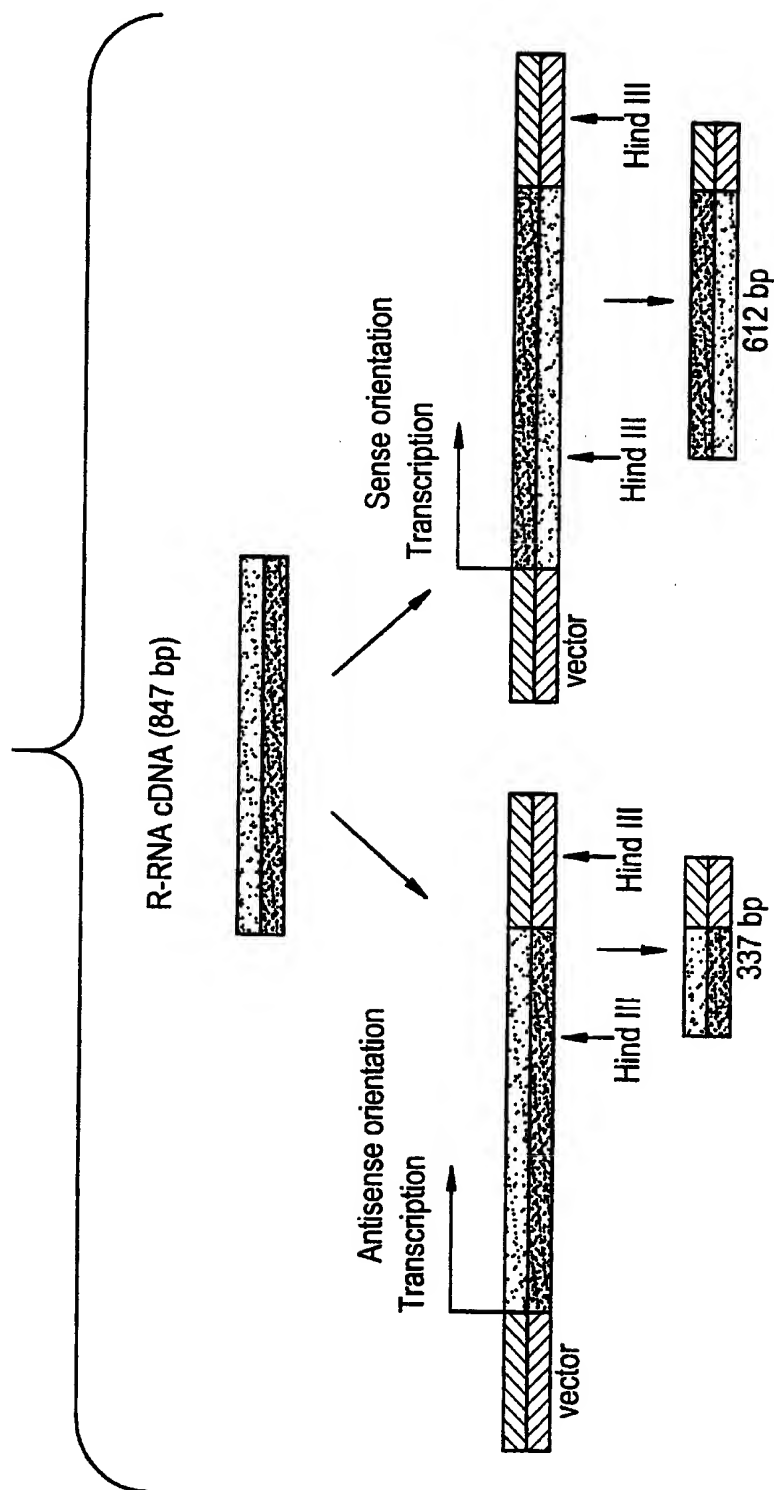


FIG.1B



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FIG. 2A



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FIG.2B

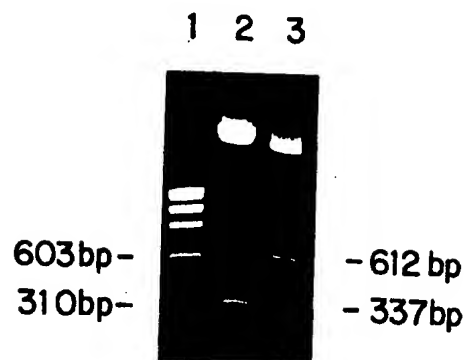


FIG.2C

	Sense probe			Antisense probe		
Antisense Txpt	●	●	●			
Sense Txpt				●	●	●
Amount [ng]	100	10	1	100	10	1
A -RNA						
A -RNA				●	●	
Amount [ng]	10	5	2.5	10	5	2.5

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FIG. 3A

1 TGCACAAGGTGACATTTTAAGAAAGTGTAGGCTTGTCGCAAGGAGTATTAGATGAAAAATAACCCAGAGAATCAATTGGTGATCTTCAA 91
 A Q G D I L R K C R L V A K E Y L D E N N P E S I G D L Q
 92 TTCAATTTGAATATCTCAGAAATAGAAAAATAATATAGTATCAGCTTCTTGAACGCTCAGACAGGAAAGTTGTCATATTAATGGATAAGCTA 181
 F N L N I S E I E N N I V S L L E R S D R K V V I L M D K L
 182 GATGAGCGGTATGAACCGGATAATATAGGAATTGGAATCAATTGCAGGTCTAGCATATGCATCTATTGAATTAATAACAAAAAGCAAAATGC 271
 D E A Y E P D N I G I G I I A G L A Y A S I E L N Q K A K C
 272 ATTCGTCCCAATAATTTTTTAAGGGATAATATATTTAGGTCGCTATCAAAAGGAAGATCCTGATTACTCGAGAAATATAGAGGGTCAAGTC 361
 I E P I I F L R D N I F R S L S K E D P D Y S R N I E G Q V
 362 ATAAGGTTGCATTGGGACTGGGCACAACTCCTAATGCTGTCAGCTAAAGAATGAAAGTAGCAATTAAGCTAGATATTGAGAAAGATCAA 451
 I R L H W D W A Q L L M L S A K R M K V A F K L D I E K D Q
 452 CGAGTTTGGGATAGATGCACAGCGGATGATCTTAAAGGGAGGAATGGTTTAAAGCGATGTTTGCAATTTACCCCTTTACCGGCCCGGAT 541
 R V W D R C T A D D L K G R N G F K R C L Q F T L Y R P R D
 542 TTACTATCATTTGTAATGAAGCTTTTTTCCGCATTTCAGAGAGAAATAGAGAAACTATCATAAACACTGACCTAGATAATGCGCCCAAG 631
 L L S L L N E A F F S A F R E N R E T I I N T D L E Y A A K
 632 TCAATTTCCATGCCAGACTTGAAGATCTCTGGAAAGAGATATCAGAAGATCTTCCCTCAATACAGGTATTAAGTAGTGCATTTTCGTAGC 721
 S I S M A R L E D L W K E Y Q K I F P S I Q V I T S A F R S
 722 ATTGAACCTGAATTAACAGTTTATACGTGCTTAAAAAATAAGAGCATCTTTCGAAATTAATCGAAGAAATGGAGATCCTAAATAACG 811
 I E P E L T V Y T C L K K I E A S F E L I E N G D P K I T
 812 TCTGAATACAGTTGTTAAAGGCAAGTGAATCCG 847
 S E I Q L L K A S G I L

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FIG.3B

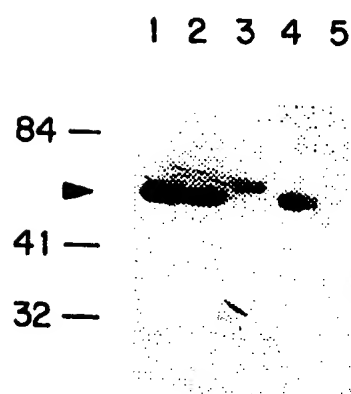
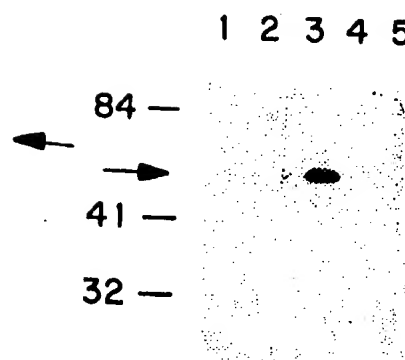


FIG.3C



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FIG.4A

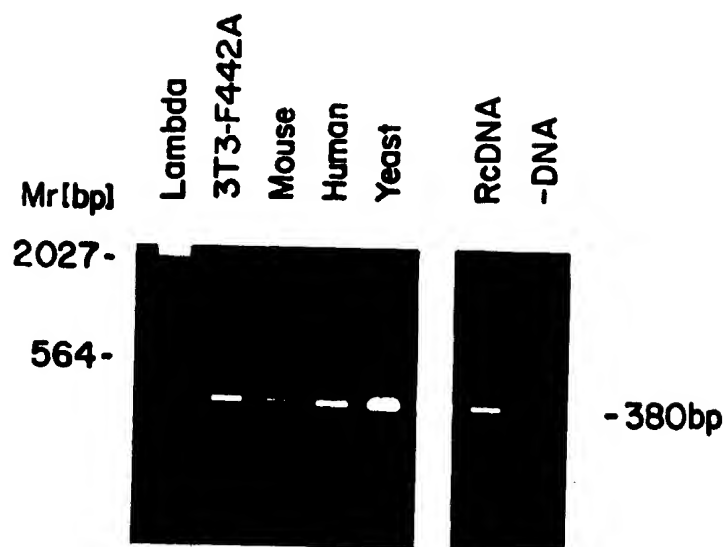


FIG.4B



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FIG.5A

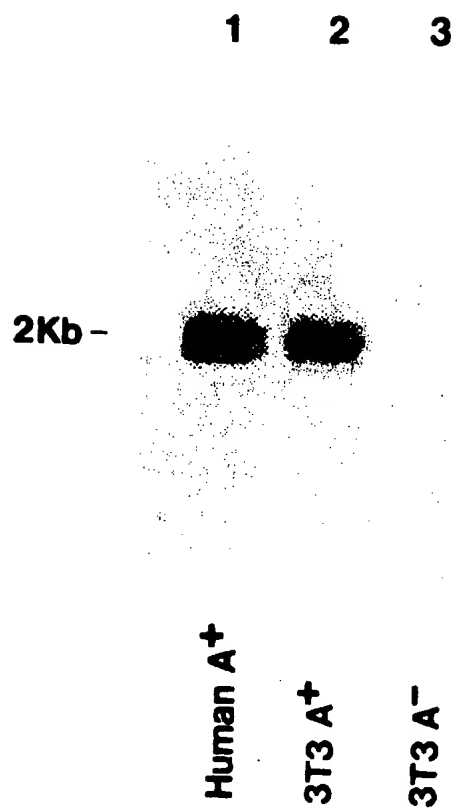
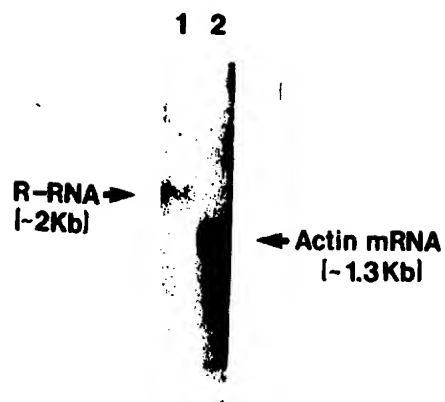


FIG.5B



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FIG.6

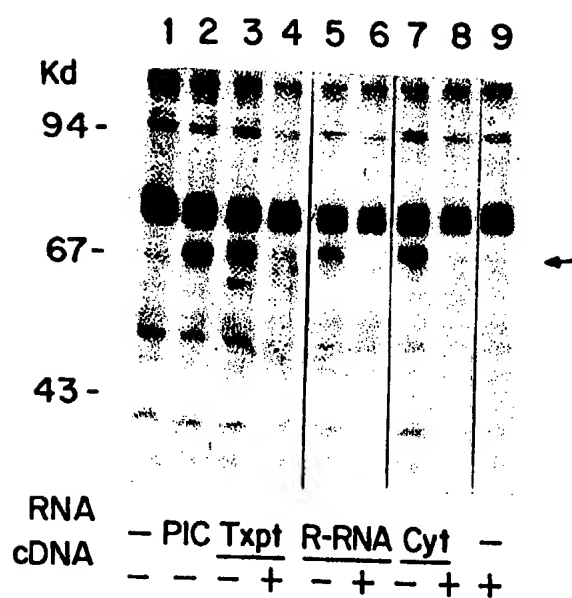
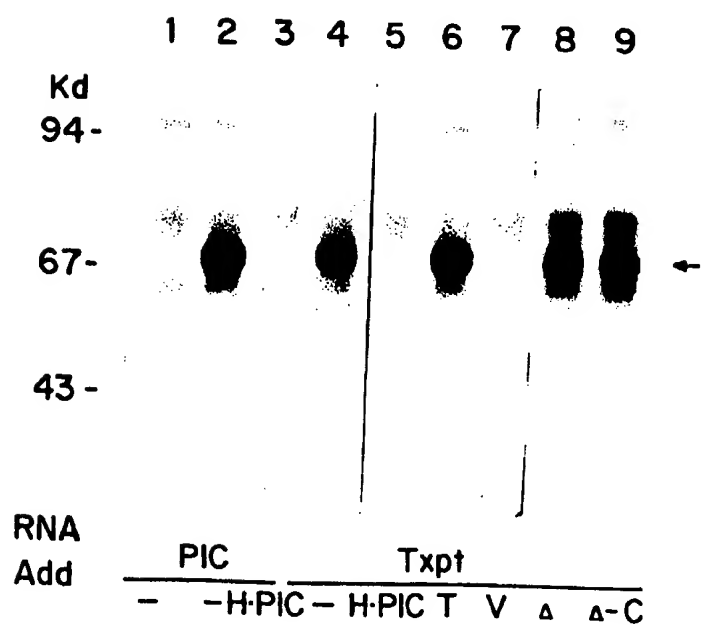


FIG.7



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FIG. 8

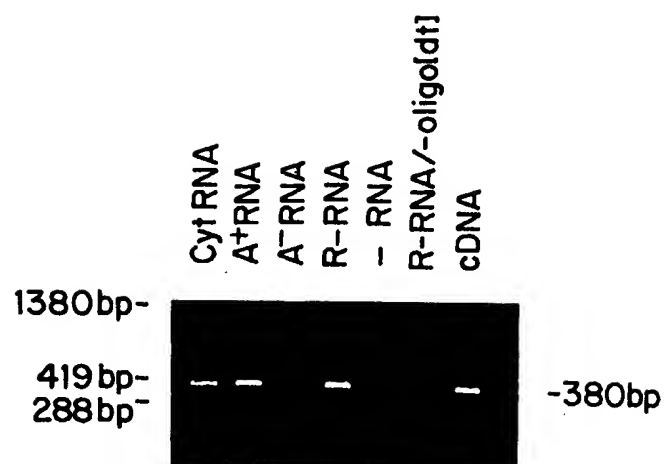
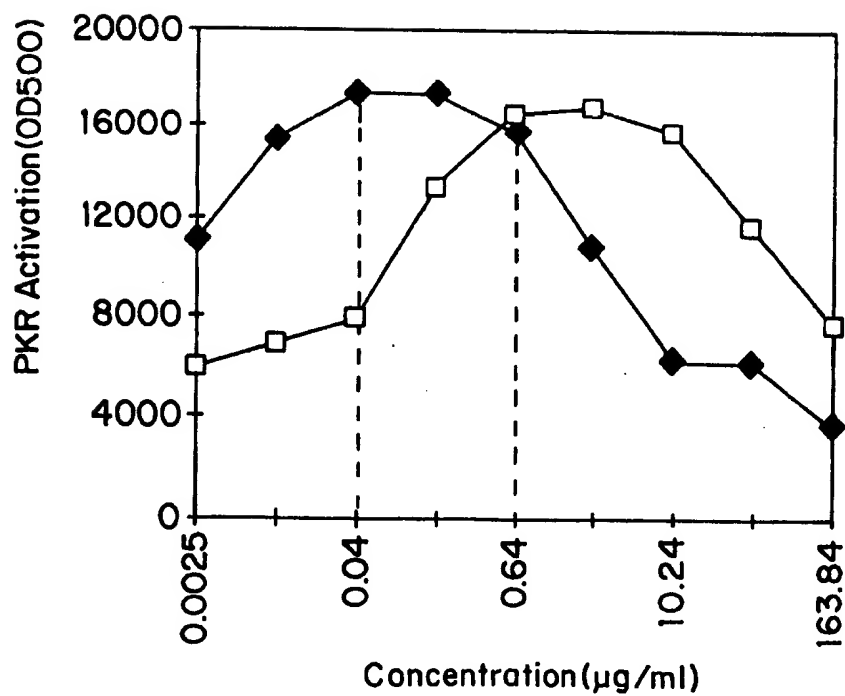


FIG. 9



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FIG. 10A

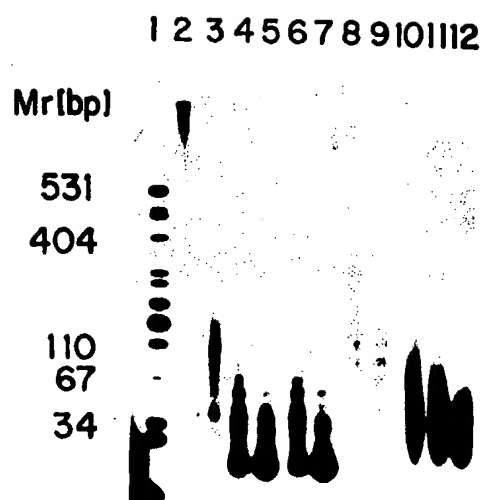


FIG. 10B

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FIG. 11A

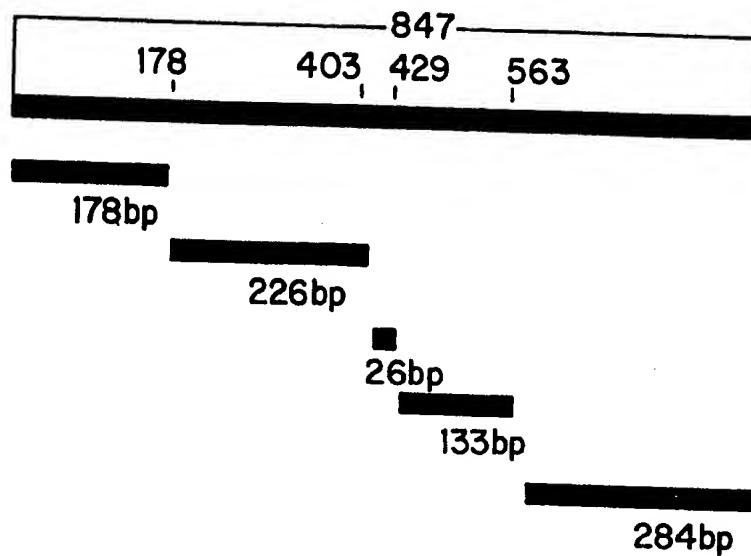
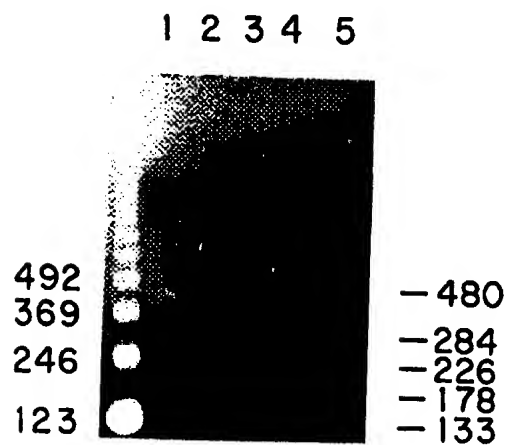


FIG. 11B



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FIG.11C

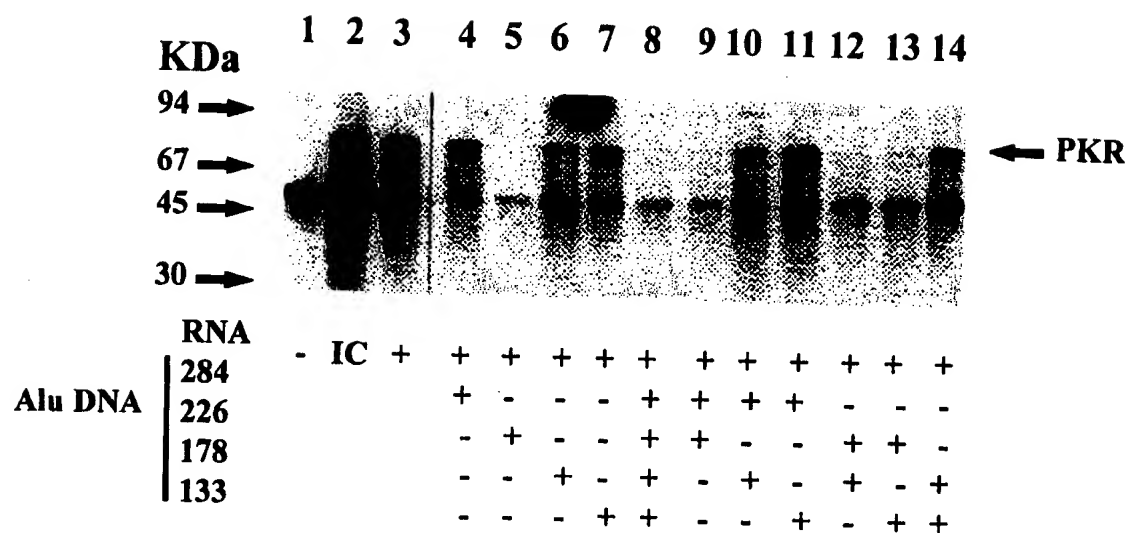
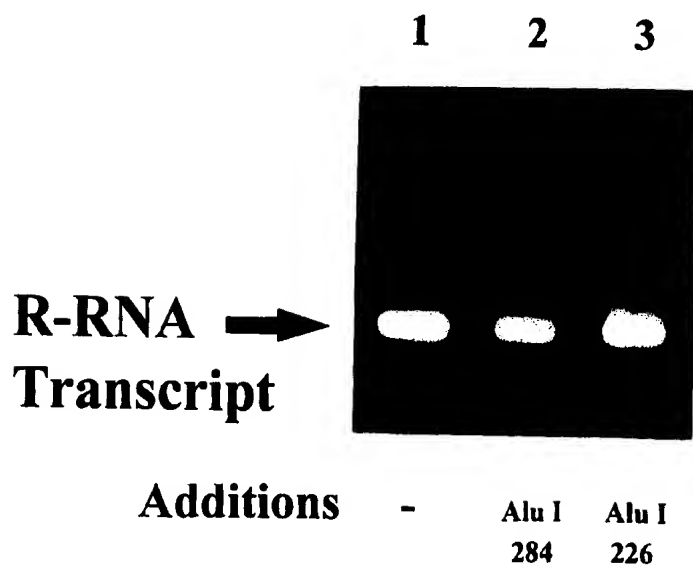


FIG.11D



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FIG.12A

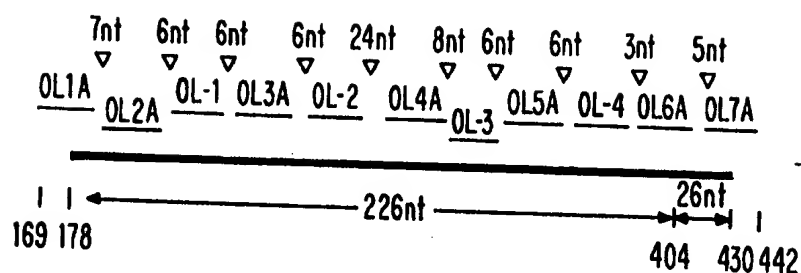
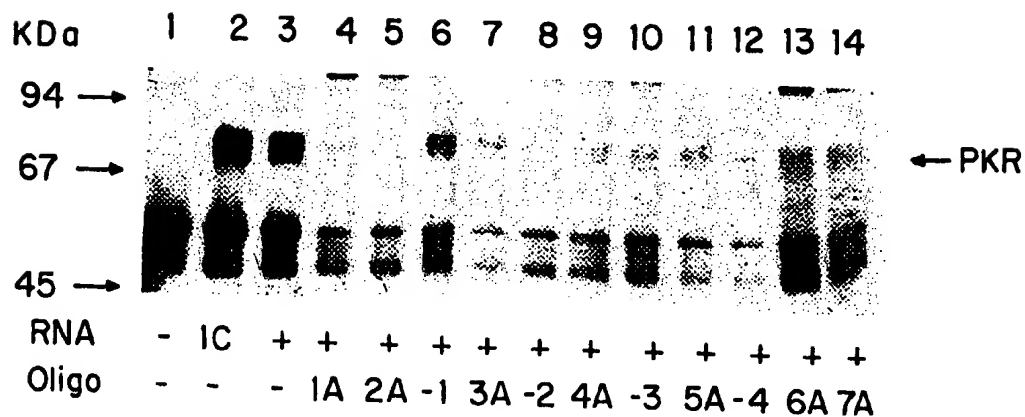


FIG.12B



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FIG.12C

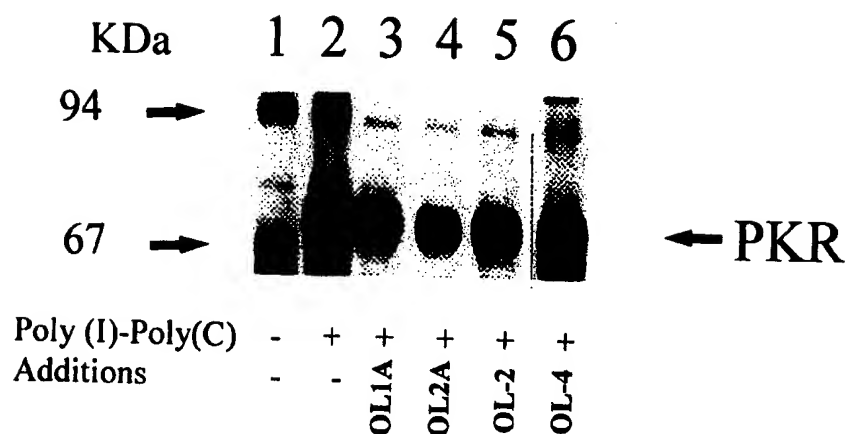
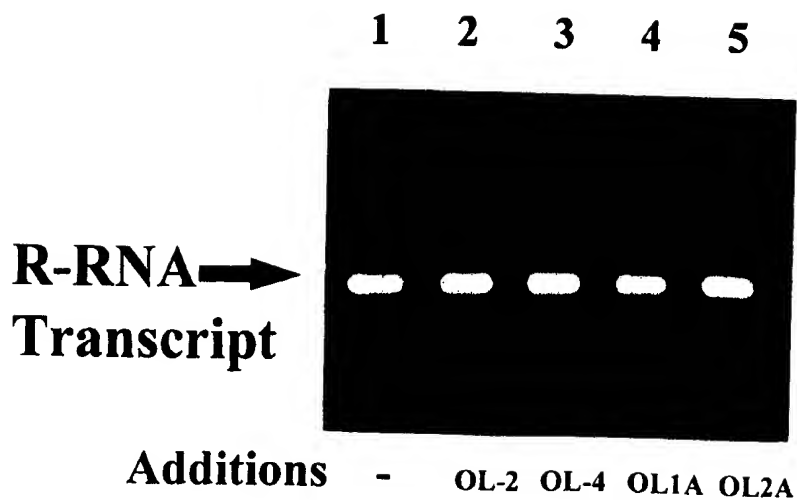


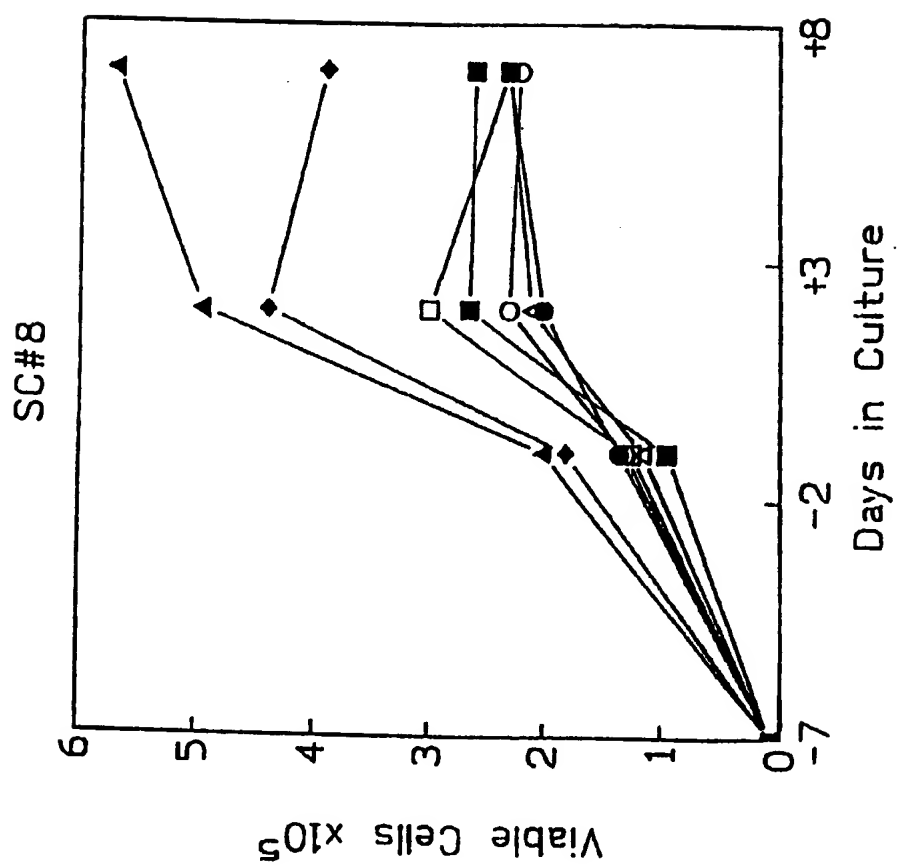
FIG.13



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FIGURE 14

3T3F442A Cell Line Cultured with 5 μ M ODN
(ODN added Day -6)



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FIGURE 15

Peripheral Blood Mononuclear Cells Exposed to $1\mu\text{M}$ ODN for 3 Day
Then Cultured in BFU-E Assay

SC#4

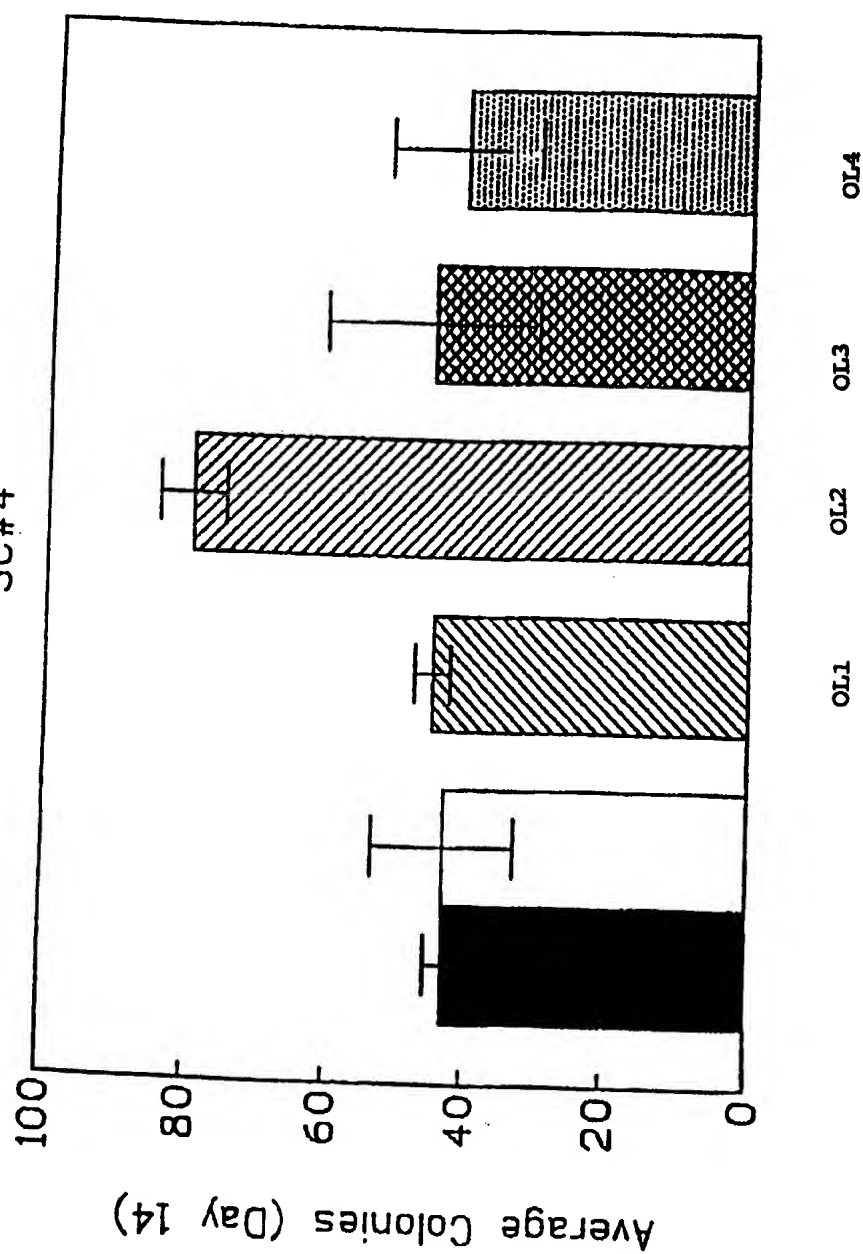
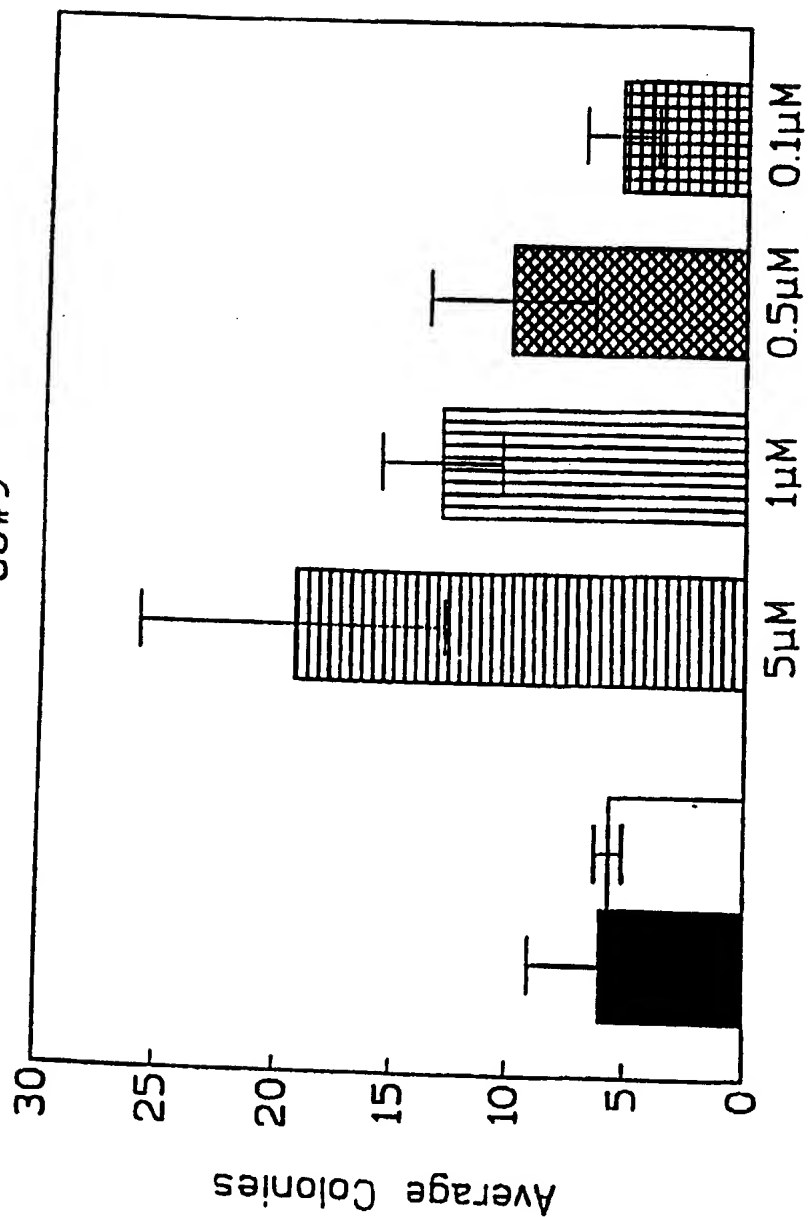


FIGURE 16

Peripheral Blood Mononuclear Cells Exposed to ODN for 3 Days
Then Cultured in BFU-E Assay

SC#9

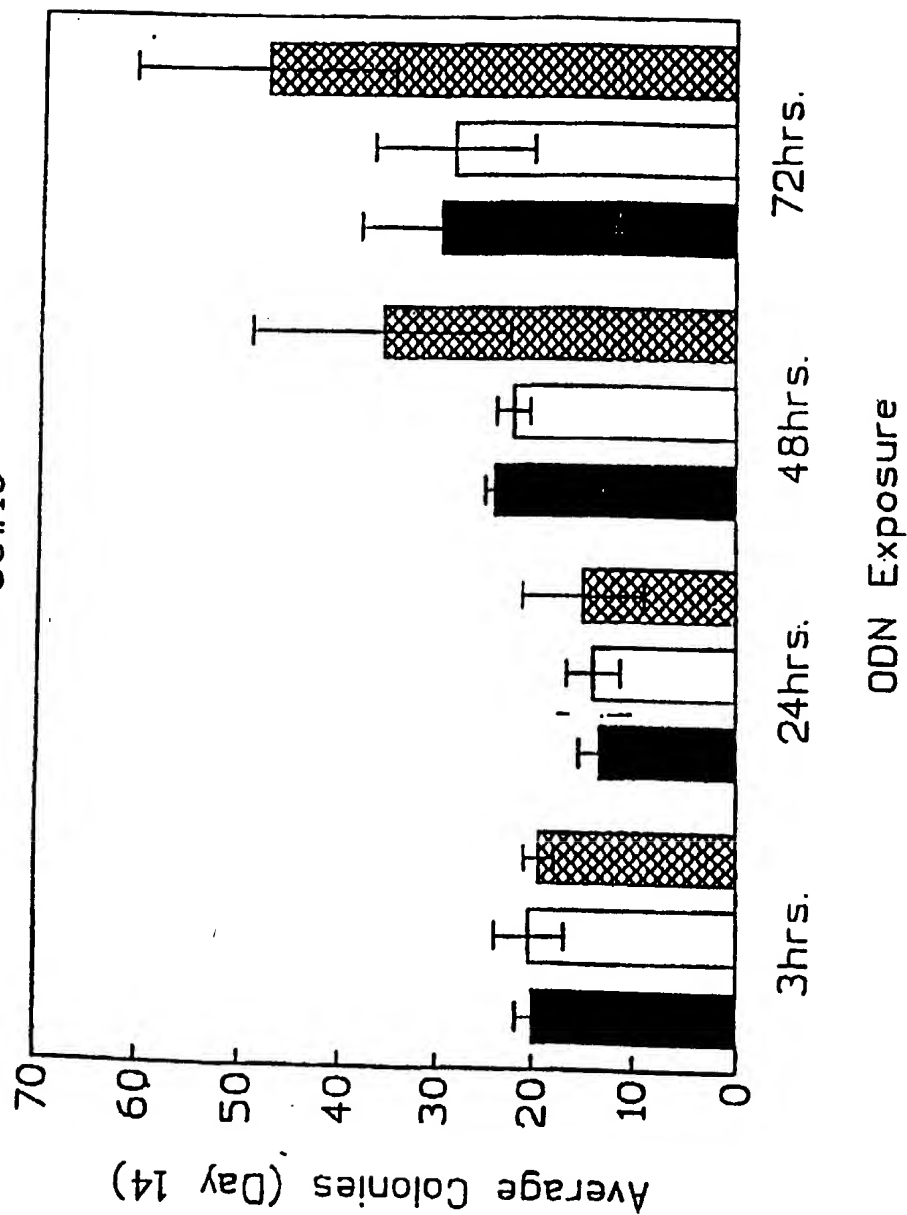


OL2

FIGURE 17

Peripheral Blood Mononuclear Cells Exposed to $1\mu\text{M}$ ODM
Then Cultured in BFU-E Assay

SC#10



Int International Application No

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 A61K31/70

B. FIELDS SEARCHED

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

29 September 1998

Date of mailing of the international search report

05/11/1998

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Bardili, W

INTERNATIONAL SEARCH REPORT

Int .tional Application No
PCT/US 98/10001

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R.A. PETRYSHYN ET AL.: "Activation of the double-stranded RNA-dependent eIF-2-alpha kinase by cellular RNA from 3T3-F442A cells" EUR. J. BIOCHEM., vol. 195, 1991, pages 41-8, XP002078958 cited in the application see in particular page 48, last paragraph ---	1,2
X	G.B. PETERSEN ET AL.: "Sequence of a transposon identified as Tn1000" DNA SEQUENCE, vol. 5, 1995, pages 185-9, XP002078959 see the whole document & DATABASE STRAND ID = ECTN1000 AC = X60200, 15 August 1991 ---	1-17
A	E. OHTSUBO ET AL.: "Identification of the region that determines the specificity of binding of the transposases encoded by Tn3 and gamma,delta to the terminal inverted repeat sequences" JAPANESE J. GENETICS, vol. 69, no. 3, 1994, pages 269-85, XP002078960 see figure 1 & DATABASE STRAND ID = ECTNPA1 AC = D16449, 31 August 1993 ---	
X	A.S. KHAN ET AL.: "Single pass sequencing and physical and genetic mapping of human brain cDNAs" NATURE GENET., vol. 2, 1992, pages 180-5, XP000601519 see abstract & DATABASE STRAND ID = HS303 AC = T03303, 22 August 1993 ---	1-6, 9-14,17
X	DATABASE STRAND ID = NITNREPSQ AC = X96404, 1 September 1996 THELMA B.K.: "Horizontal transfer of sequences identical to an integral fragment segment of bacterial transposon Tn1000 is widespread in the genome including humans" XP002078962 ---	1-17

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INTERNATIONAL SEARCH REPORT

Int. l. Application No.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MAITRA R.K. ET AL.: "Catalytic cleavage of an RNA target by 2-5A antisense and RNase L*"</p> <p>J. BIOL. CHEM., vol. 270, 1995, pages 15071-5, XP002078961</p> <p style="text-align: center;">-----</p>	